LIPID TRANSPORT
AND ITS POSSIBLE RELATIONSHIP TO
EXPERIMENTAL ATHEROSCLEROSIS

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This thesis is my own work. Any collaboration with colleagues is indicated in the text.

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PREFACE

A rather wide area has been covered in this thesis and it is hoped to explore some of the facets in more detail in the future. The general pattern however has stemmed from the interest of the Department of Experimental Pathology, and especially of Professor F.C. Courtice, in the size and make-up of lipoproteins as regards their significance in relation to atherosclerosis.

The emphasis in this work has been on the lipoproteins of thoracic duct lymph before entry into the blood stream. This was made possible by a relatively simple technique of thoracic duct cannulation in the rabbit developed in collaboration with Dr Zilversmit. The measurement of the size of lipoproteins was determined by electron microscopy with the help of Dr Cliff.

The examination of particulate lipid in artificial fat emulsions was also undertaken because of the increasing use of these substances in clinical medicine. Dr Håkansson of Vitrum A.B. of Stockholm, Sweden, was of assistance in supplying material and data; while Dr Schoefl was a valued critic.

Some aspects of the fate of lipoproteins after entry into the blood stream have also been studied but need to be examined in further detail. The development of experimental atherosclerosis in the rabbit has been looked at to see if it is related to the diet and size of lipoproteins entering the blood stream from the thoracic duct lymph.

Although the statement is true that all experimental procedures were carried out by me, it must be emphasised that my many teachers and advisers included not only my supervisor, Professor Courtice, who was an inspiration, leader and colleague, but also Dr Zilversmit, who was visiting the department during my formative first few months. The many other academic members of the John Curtin School were always of assistance, as were Mrs McLeod and Mrs Smith in the school library.

The technical assistance was of high calibre. Mr Hill prepared the microscopic sections for light microscopy, Mr Westen and his staff in photography were always willing to help, Mr Harding made materials easily available, and Miss Nangle and Mrs Swan were efficient typists. I also wish to acknowledge assistance given by Miss Dempsey, Miss Lloyd, Miss Soldan, Mrs Borucinski and Mr Pryke.

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INTRODUCTION

Coronary heart disease and myocardial infarction are usually associated with vessels narrowed with advanced atherosclerosis. Atherosclerosis is a disease of arteries characterised by atheromatous plaques. These plaques are focal in distribution and consist of fibrocellular thickenings of the intima containing varying amounts of lipid (Strong and McGill, 1962; Schwartz and Mitchell, 1962; Friedman, 1963). In 1958 the World Health Organization defined atherosclerosis as a condition characterized by a variable combination of changes of the intima of arteries consisting of focal accumulations of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits.

In man the origin of the lipid in the atheromatous lesions and the mechanisms concerned in its deposition have led to a considerable amount of research in the field of lipid transport and metabolism. The evidence suggests a positive correlation between the levels of certain lipids in the serum, the proportion of total calories provided by dietary fat, and mortality from coronary heart disease (National Heart Foundation of Australia, 1967).

It is well known that the lipids in body fluids are transported as a broad spectrum of lipoprotein complexes which in recent years have been separated and analysed by ultracentrifugal and other methods (Lindgren and Nichols, 1960). Isotope labelling has shown that individual lipids in the lipoprotein complexes, or the lipoproteins themselves in the serum exchange with those in the atheromatous lesion (National Heart Foundation of Australia, 1967). Other experiments indicate that the intact lipoproteins, like all other proteins in the plasma, are continuously circulating throughout the extracellular fluid, leaking from the small blood vessels into the tissue fluid to be returned to the blood stream by the lymphatic vessels (Courtice, 1968). The mechanisms of lipid deposition in the atheromatous plaque of the artery, therefore, may be concerned not only with the levels of certain lipids in the serum, such as cholesterol and triglycerides, but also with the size and structure of the lipoproteins in which the lipids are carried.

In animals lesions in the arterial intima do occur naturally, but are not so extensive as in man, nor are they generally so rich in lipids (Sandler and Bourne, 1963; Roberts and Strauss, 1965). Extensive lipid-

containing lesions have, however, been produced experimentally in animals by raising the levels of serum cholesterol. In rabbits this may be achieved merely by the addition of cholesterol to the diet (Anitchkow, 1933). This experimental model has been used extensively to study the mechanisms of lipid involvement in the arterial lesions.

The study of the movement of dietary cholesterol from the gut lumen to the atheromatous plaque is very complex, but it does involve the formation, transport and breakdown of large lipoprotein complexes consisting essentially of insoluble triglyceride and cholesterol stabilized in a watery medium by phospholipid and protein. This thesis deals mainly with the study of these lipid particles or large lipoprotein complexes in both the thoracic duct lymph and blood stream of rabbits fed varying quantities of triglyceride and cholesterol. Emphasis has been placed on the morphology and size distribution of particles in relationship to their lipid composition. It will be shown that as the dietary fat load increases the particles entering the blood stream from the thoracic duct become larger and the possible fate of these particles will be studied with particular reference to hypercholesterolaemia and

the production of experimental atherosclerosis in the cholesterol-fed rabbit.

1. Fat absorption

(i) Discovery of the lacteals and chylomicrons

Lymphatic vessels are difficult to see in most tissues but may readily be observed when the tissue is injected with a visible material such as a dye solution. In the living animal the vessels of the small intestine are naturally "injected" with fat after a fatty meal and appear white. This function of the lymphatic vessels led to their observation as early as 300 B.C. by Herophilus and Erasistratus in the famous Alexandrian Medical School. Galen regarded these vessels as veins and it was not until the Renaissance, when independent thought and experimental study again prevailed, that the lacteals were properly described. Asellius in 1622, after examining the viscera of a living dog, wrote:

> ...I suddenly beheld a great number of cords as it were, exceedingly thin and beautifully white, scattered over the whole of the mesentery and the intestine, and starting from almost inumerable beginnings....having laid hold of a very sharp scalpel, I pricked one of those cords and indeed one of the largest of them. I had hardly touched it, when I saw a white liquid like milk or cream forthwith gush out... (Drinker, 1942).

Asellius thought that the lacteals drained to the liver and it was Pecquet, a little later in 1650, who

traced these vessels through the receptaculum and thoracic duct to the great veins of the neck. In his Experimenta Nova Anatomica of 1651 he wrote:

> I, by the leave of so great men, would say that not any of them by a particular inquest have searched the Lurkings of these Lacteal Veins within the Thorax. But I believe this is rather to be attributed to their misfortune than negligence, because none of them knew that the Chyle was not derived to the Liver, nor to the Vena porta, nor the Vena cava near the Emulgents, as the received errour held forth: but, which in dissection may be seen to any man more clear than the light, From the Guts to a certain RECEPTACLE of that bigness, which will fill up the interstitium between the Lumbar Muscles, at least in Beasts.
>
> Now this receptacle above the Vertebers of the Lyons receives the Liquor of the Milkie Veins spread in the Mesentery, and rendreth it again by those Milkie Veins, which being hid within the breast, in a continued passage run to the Subclavial venal branches, till within the ascending stem of the Vena Cava about the External Jugulars, being mixt with the blood and running in one and the same Chanel, it throws its self headlong into the Whirlpool of the Heart,... (Drinker, 1942).

The discoveries of the lymphatic vessels of the small intestine and of the thoracic duct, the transport system of most of the lipids from the gut to the blood stream, marked the beginnings of the scientific studies of fat transport and metabolism.

Another landmark in the history of fat absorption came a century later in 1774 when William Hewson, a pupil of William Hunter, wrote his treatise on the

lymphatics. He postulated that the lacteals were connected with the intestinal lumen by pores and he speculated on the mechanisms which caused these pores to open or close. He thought that intestinal chyle was absorbed through these pores as a whole. Under the microscope he saw many exceedingly fine particles in the lymph from the lacteals. These were more fully described by Gulliver in special papers and editorial notes upon Hewson's work. Gulliver in note LXXXIII on Hewson's paper wrote:

> Before Hewson's time, it was a popular opinion that lacteals begin by open mouths in the villi of the intestines, and this view continued to be generally entertained until lately. Mr Sheldon, after stating that these mouths were discovered by Liberkühn, declared that 'the ampullulae, with their orifices, are to be considered as the beginning of the lacteal vessels'. Mr Cruikshank figured what he conceived to be orifices. But Professor Müller found that mercury injected into the lacteals does not escape from the surface of the mucous membrane of the gut, and therefore he concluded, with Rudolphi, that the villi are not perforated at their extremity. In short, it is now well known that the radical extremities of the lacteals form loops, or closed passages in the villi.

Gulliver and Hewson showed that the small particles were the same in lacteal fluid or chyle and in the blood stream, and called them "the molecular base of the chyle", and described their Brownian movement. They also noted that the milky fluid contained much fat for if dried on a filter paper it left a grease spot. Edmunds

(1877), with the dark field microscope, noted blood contained fine particles and said that "the blood seems like a wholly new substance with multitudes of glancing particles which look like motes in a sunbeam".

Simon Henry Gage in 1920 again described these particles in human and animal blood and wrote,

> As these particles almost surely get into the blood by way of the chyle vessels and thoracic duct, I have named them Chylomicrons, that is, microscopic bodies from the chyle.

However in the better known paper of Gage and Fish (1924) the thesis originally propounded by Hewson that some of the fat particles of the blood might come from remobilised fat reservoirs of the body, as well as from ingested food or chyle, was investigated, and on pages 6 and 27 Gage and Fish also used the term chylomicrons for particles not originally derived from the chyle. They studied in various animals the sites of absorption of fat from the small bowel, the transport of chylomicrons through the lacteals and thoracic duct, their presence in the blood stream and finally their disappearance from the circulation.

(ii) <u>Transfer of triglyceride from intestinal lumen to lacteals</u>

It is well established that fat is ingested and transported from the gut in the lacteals mainly as

triglyceride. The intermediate mechanisms concerned in its passage through the cells of the intestinal mucosa have, however, been debated for more than a century. Pflueger in 1900 put forward the idea that it was necessary for triglycerides to be hydrolyzed to fatty acids and glycerol before absorption. Verzár and McDougall in 1936 concurred with this hypothesis and suggested that under the control of adrenal cortical hormones absorbed glycerol and fatty acids were reunited in the mucosal cell to form phospholipid, an essential intermediate in the resynthesis of triglyceride.

Frazer first in 1938 and in more detail in 1943 brought forward his partition theory of fat absorption in which he noted a differentiation in the absorption of olive oil and oleic acid in the rat. He suggested that complete hydrolysis of triglyceride in the intestinal lumen was not essential for fat absorption and that un-hydrolysed triglyceride could be absorbed by mucosal cells and from there reach the blood stream via the lacteals and the thoracic duct. Fatty acids, however, were not resynthesised to triglycerides by the mucosal cells but entered the blood stream directly to be carried by the portal vein to the liver. This latter

observation conflicted with the earlier findings of Gage and Fish (1924) and later work by Winter and Crandall (1941) and Bloom et al (1950). Bergström et al (1954) and Simmonds (1955a) also failed to confirm Frazer's findings. Meanwhile in 1952 Frazer modified his original hypothesis and only stressed that complete hydrolysis in the gut lumen was not a pre-requisite for the absorption of triglycerides but felt that intraluminal emulsification of fat was of importance.

In the last twenty five years experimental evidence has led to the micellar theory of fat absorption. The first direct proof that some glyceride was absorbed unhydrolysed came in 1952 when Bernhard, et al, and Reiser, et al isotopically labelled glycerides both in their glycerol and fatty acid fractions. Hofmann and Borgström (1962, 1964) using the ultracentrifuge fractionated the luminal content of the small intestine after a fatty meal into an upper phase containing an emulsion of triglyceride and a lower water soluble or micellar phase containing bile salts, fatty acids, monoglycerides and a little di- and triglyceride.

It is now felt that dietary fat mixes in the duodenum with bile, which contains bile salts, phospholipids, cholesterol and mucopolysaccharides, to

form an emulsion of small fat droplets. The lipase of pancreatic juice then acts on the emulsified triglyceride, especially at the 1 and 3 bonds of the glycerol fatty acid complex, to release fatty acids, glycerol-2-monoglycerides and small quantities of diglycerides. The conditions are then ready for a water soluble micellar state to be formed between the bile salts, fatty acids, mono and diglycerides within the gut lumen. The bile salts can be regarded as carriers of the products of hydrolysis from the emulsion phase of the lipid to the brush border of the mucosal cells.

There are probably two stages in the absorption of the intraluminal products of fat digestion by the mucosal cells of the small bowel. The first is a passive diffusion of these products into the cell and the second their active re-esterification within the cell to form triglyceride.

The bile-salt micelle comes in contact with the brush border which passively accepts the fatty acids and monoglycerides leaving behind bile salts to accept further products of triglyceride hydrolysis in the gut lumen (Playhoust and Isselbacher, 1964a and b; Dietschy, 1968). However with the electron microscope Palay and Karlin (1959) noted vesicles in the terminal web of the

mucosal cells and postulated an active pinocytosis of triglyceride or its breakdown products from the bowel lumen but in 1963 Palay and Revel, although still convinced of the presence of pinocytosis, admitted passive diffusion might also play a part. Most studies, nevertheless, suggest that the first step in fat absorption by the mucosal cells is a passive, energy independent diffusion into the cells (Sjöstrand, 1963; Cardell et al, 1965; Strauss, 1966).

The second step in the process of fat absorption by the mucosal cells is energy dependent and consists of the resynthesis of triglyceride. Palay and Karlin (1959) noted the close relationship of the endoplasmic reticulum of the mucosal cells to the formation of chylomicrons, and their discharge into the extracellular fluid from the edge of the mucosal cells. Senior and Isselbacher (1960, 1962) by homogenising and ultracentrifuging mucosal cells found the microsomal fraction contained enzyme systems which were heat labile and were capable of re-esterifying fatty acids and monoglycerides to triglycerides. In this same fraction were systems capable of synthesising phospholipids and the ribosomes necessary for the synthesis of the protein found in chylomicrons.

A third step may be suggested by the findings of Redgrave (1967) who noted that mucosal cells at the base of the microvilli could passively absorb fatty acids and monoglycerides, and resynthesise these to triglyceride; but not until the cells matured and were situated further towards the apex of the microvilli could they form chylomicrons.

It seems likely that the resynthesis of triglyceride, the synthesis of phospholipid needed to coat and emulsify the triglyceride, protein synthesis and the re-esterification of cholesterol, all the steps necessary for the formation of the chylomicron, take place in the endoplasmic reticulum of the mucosal cell. The completed chylomicrons are then discharged from the side of the cell to the extracellular fluid and from there to the lacteals and thoracic duct.

(iii) Absorption of dietary cholesterol

Pribram (1906) noted an elevation of serum cholesterol after feeding rabbits cholesterol. This observation together with the later observations of Anitschkow and Chalatow (1913) and Anitschkow (1933) of lipid deposition in the arteries of cholesterol-fed rabbits have resulted in much work on cholesterol metabolism in man and animals. Goodman (1965) in a review

of cholesterol ester metabolism stated that a full and detailed understanding of cholesterol absorption will require advances in our understanding of the processes of chylomicron formation and their entrance into the intestinal lymphatics. These will probably come from a combined biochemical and morphological approach to this problem.

Mueller (1915) proved by means of thoracic duct fistulae in dogs that dietary cholesterol reached the blood stream via the thoracic duct. This finding was confirmed by Chaikoff et al (1952) by means of feeding radioactive cholesterol to rats.

In order to be absorbed dietary cholesterol must first be in the unesterified form. Pancreatic cholesterolesterase in the presence of bile releases free cholesterol in the lumen of the intestine which, like the products of triglyceride digestion, enters a micellar phase with bile acids and free fatty acids (Hofmann and Borgström, 1962; Simmonds et al, 1967). The free cholesterol is then absorbed by the mucosal cell and Cook and Thomson (1951) have shown that up to 34, 47 and 77 per cent of dietary cholesterol is absorbed in the rat, guinea pig and rabbit respectively.

Ganguly and Murthy (1963) stated that the cholesterol content of mucosal cells was constant whether the animal was starving or absorbing cholesterol. The cholesterol of the cell was in the unesterified form mainly associated with the microsomal fraction of the cells. Presumably during cholesterol absorption the same amount of cholesterol enters the mucosal cell from the gut lumen as leaves the cell either into the extracellular tissues or back into the gut lumen. The cholesterol of thoracic duct lymph, however is mainly in the esterified form (Vahouny and Treadwell, 1957; Zilversmit, 1968b). The free cholesterol in the cell is re-esterified before reaching the thoracic duct probably under the influence of a pancreatic esterase (Swell et at, 1950) in the presence of bile (Wilson and Reinke, 1968).

Cholesterol, like triglyceride from the intestine, is transported to the blood stream in the thoracic duct lymph as a constituent of lipoproteins or chylomicrons (Zilversmit et al, 1967). This will be more fully discussed in a later chapter, as will its fate and possible pathological significance.

(iv) Role of phospholipid in triglyceride and cholesterol transport

Fatty acid esters of cholesterol and glycerol are insoluble in water. Another group of fatty acid esters

transported in lipoprotein complexes, the phospholipids, act as stabilizers (Elkes and Frazer, 1943). Fredrickson *et al* (1967) suggested that one cannot escape the intuitive conclusion that phospholipids are mainly in plasma to function as "biological detergents". The high surface activity of phospholipids promotes stability at the oil water interfaces represented by the lipoproteins and their interactions with plasma.

It is doubtful if the phospholipids in the intestinal lumen, whether dietary in origin or from the bile, are absorbed as such by the mucosal cells. Rather they play a part in the intraluminal emulsification of triglyceride and are themselves broken down by pancreatic phospholipase. The fatty acids of thoracic duct phospholipids differ from the fatty acids of the diet or of the plasma (Whyte *et al*, 1963; Zilversmit, 1968a) and only a small proportion of absorbed fatty acid reaches the lymph as phospholipid (Bloom *et al*, 1951) suggesting that phospholipids stabilizing the chylomicrons from the intestine are synthesised in the endoplasmic reticulum of the mucosal cell (Gurr *et al*, 1963; Senior and Isselbacher, 1962; Zilversmit, 1968a). This subject will be further discussed in a later chapter.

2. Lipoproteins : their analysis and characterization

As mentioned previously the lipids in lymph and serum are transported as lipoprotein complexes. Although fat particles or "chylomicrons" had been observed in lymph and serum earlier, the first description of a lipoprotein of constant composition was made only as recently as 1929 by Macheboeuf. He reported that at least certain of the serum lipids existed in some type of chemical association with proteins and designated these complexes as lipoprotein coenapses. Since this time the characterization of the lipoproteins has been advanced mainly by the introduction of the ultracentrifuge.

(i) Fractionation of lipoproteins by ultracentrifugation

Whereas most proteins have densities from about 1.26 to 1.38 g/ml the lipoproteins are lighter with densities ranging from about 0.93 to 1.15 g/ml depending on the relative proportions of lipid and proteins. From 1935-1945 the ultracentrifugal studies of serum by McFarlane and Pedersen led to the suggestion of the existence of a labile lipid protein complex in human serum. Meanwhile the rapid progress in chemical plasma protein fractionation by Cohn, Oncley, Edsall

and associates led to the identification of two major lipid containing fractions in human serum called the α- lipoprotein consisting of about 35 per cent lipid and 65 per cent protein and the β- lipoprotein fraction of about 75 per cent lipid and 25 per cent protein. Oncley and associates pointed out that essentially all the serum cholesterol could be accounted for in one or other of the lipoprotein fractions (cf. Gofman et al, 1954).

A rapid advance in the separation and classification of the serum lipoproteins resulted from the studies of Gofman and his colleagues with the analytical ultracentrifuge. It was revealed that some of Pedersen's labile x protein was indeed low density lipoprotein. (Gofman et al, 1949). Subsequent work has shown a whole series of lipoproteins in human serum differing from each other in such properties as hydrated density, molecular weight and chemical composition. Early in these studies it was shown that essentially all of the serum cholesterol, both free and esterified, the glyceryl esters and the phospholipids existed in one or other of the members of the ultracentrifugally defined lipoprotein series. The fraction of the serum remaining after

ultracentrifugation contained no cholesterol, phospholipids or glyceryl esters.

The ultracentrifugal characterization of the serum lipoproteins as studied by Gofman is based upon two major operations. First a preliminary quantitative separation of the lipoproteins from the serum proteins is achieved by ultracentrifugation in a medium the density of which is so adjusted as to cause flotation of those lipoproteins of hydrated density lower than that of the medium, and sedimentation of the proteins which are of greater density. Next the isolated lipoproteins are characterized by analytical ultracentrifugation under the specified conditions used. For convenience an arbitrary designation has been employed which segregates lipoproteins into two major groups, the 'low density' lipoproteins including all species of hydrated density less than 1.04 g/ml and the 'high density' group, including three species of hydrated densities 1.05, 1.075 and 1.145 g/ml. The type of ultracentrifugal procedure most commonly used involves bringing the solution density up to 1.063 g/ml with sodium chloride and subsequent ultracentrifugation at 26°C. Under such conditions members of the low density spectrum of lipoproteins undergo flotation with rates

between 4 and 40,000 Svedbergs. Ideally it would be desirable to convert observed flotation rates to the classically used '$S_{20\ W}$' values described by Svedberg. However, conversions to such values presume the availability of precise physical data such as solution viscosity and the hydrated densities of every lipoprotein species encountered. Such data are not available at present. A practical alternative that has been utilized extensively involves the characterization of the human serum lipoproteins in terms of observed flotation rates under specified conditions of solution composition, density and temperature. Flotation rates have been measured in S_f units, or Svedbergs of flotation. This unit is identical with the Svedberg unit, except that the subscript $_f$ implies flotation. The use of S_f units provides a positive scale of values for species undergoing flotation.

The S_f unit equals a flotation rate of 1×10^{-13} cm/sec/dyne/g at $26^{o}C$. For purposes in this thesis and as used in most current literature it is used for lipoproteins undergoing flotation in a medium of 1.063 g/ml density achieved by the addition of sodium chloride to serum. This can also be expressed as $S_{f1.063}$.

In practice the existence of a very large number of human low density lipoproteins precludes the measurement of the serum concentration of individual lipoproteins of a specified flotation rate. It has become customary therefore to measure lipoprotein concentrations in terms of the sum of concentrations of all the lipoproteins between any two specified flotation rate limits. Thus the sum of concentrations of all lipoproteins present between the limits of S_{f10} and S_{f20} is measurable and referred to as S_{f10-20}. The actual choice of flotation rate limits for lipoprotein concentration measurements is arbitrary and depends on the interest of a particular physiological study (cf. Gofman et al, 1954).

Havel, et al (1955) introduced a more simple method for separating lipoproteins into different groups of S_f values using only the preparative ultracentrifuge and different density media. The method developed by these workers is essentially that used in this thesis as described in the methods. In principal, the method depends on the selection of a density medium at which there are few lipoproteins of the same density, then prolonged ultracentrifugation will eventually cause flotation of all the less dense lipoprotein species and

precipitation of all the more dense. The subnatant may again be made more dense by the addition of a heavier salt solution and the lipoproteins in that fraction further separated. By this method relatively large volumes of serum may be separated into ranges of S_f value for chemical analysis. It again must be pointed out that the lipoproteins in each fraction represent a series of lipoproteins within a range of S_f values.

(ii) The use of electron microscopy in the study of lipoprotein complexes

Another fairly recent development has been the use of the electron microscope for the study not only of the lipoproteins (Hayes and Hewitt, 1957; Kay and Robinson, 1962; Courtice and Garlick, 1962; Garlick, et al, 1965; Bierman et al, 1966; Schoefl, 1968; Fraser et al, 1968; Salpeter and Zilversmit, 1968), but also the processes of the passage of lipid from the gut lumen to the lacteals (Palay and Karlin, 1959; Ashworth et al, 1960; Casley-Smith, 1962; Jones et al, 1963; Sjöstrand, 1963; Cardell et al, 1965; Ashworth and Lawrence, 1966; Strauss, 1966) and the uptake and synthesis of

lipoproteins by the liver (Ashworth et al, 1960; Jones et al, 1966) and other organs including the arterial wall (Parker and Odland, 1966).

One of the main themes in this thesis is the importance of the size of lipid complexes entering the blood stream and their subsequent fate. The development of an electron microscopic method for measuring lipid particle sizes will be discussed at length in the chapter on methods and from these measurements and the comparative lipid composition of the lipid particles deductions will be drawn as to the structure of particulate lipid.

3. The dynamics of lipoprotein complexes in lymph and serum

The fractionation and physical characterization of lipoproteins are initial steps essential for the study of their chemical composition and metabolism.

(i) Composition - lipid and protein analysis

Improved methods of lipid analysis have enabled measurements of the three major lipids of lipoproteins, namely triglyceride, cholesterol and phospholipids to be made. Some of these methods have been used in this thesis and will be described later.

The study of the protein component of the lipoprotein complexes has not been attempted in this thesis since the main effort has been directed towards the larger complexes or chylomicrons which contain relatively little protein (Dole and Hamlin, 1962). Methods such as electrophoresis, immunochemical techniques, amino acid patterns and radioactive labelling of the protein complex have been used to study their dynamics such as sites of formation, transport, metabolic fate and the structure of lipoproteins (Putnam, 1960; Wissler and Kao, 1962; Oncley, 1963; Walton, 1967; Scott and Winterbourn, 1967).

Fredrickson et al (1967) suggested that one might think of the lipoproteins as carriers of lipid since the half-life of the protein component is much longer than the half-life of the lipid components. Another interesting method in the study of lipoproteins has been to follow 'nature's experiments' in which certain groups of lipoproteins are congenitally absent resulting in deranged lipid transport and metabolism.

(ii) Isotopic labelling of lipoprotein complexes

Although labelling by means of dyes had been previously used (Gage and Fish, 1924) the advent of radioactive labelling techniques has made possible the

detailed study of individual components of the lipoproteins.

Many of the recent advances in the understanding of the absorption, synthesis and metabolism of triglycerides, cholesterol and phospholipids, as previously described, have been a result of the use of this technique. The study of lipoproteins labelled in their different components has led to the realisation of the complex dynamics concerned in the transport and interaction of the different lipids and proteins.

Radioactive labelling has permitted the tracing of lipoproteins and their components into various tissues of the body and the arterial wall. By these methods workers have studied cholesterol and other components of lipoproteins within atheromatous lesions, and also the metabolism of lipids within the artery wall (Chernick et al, 1949; Biggs and Kritchevsky, 1951; Charman and Lipsky, 1967; Hollander and Kramsch, 1967; Day, 1967; Whereat, 1967).

Radioactive methods will be used in this thesis to study the breakdown of chylomicrons to smaller lipid complexes by clearing factor lipase in vitro and the study of the disappearance of labelled cholesterol and triglyceride in lymph chylomicrons from the blood stream.

(iii) Exchanges of the lipoprotein complexes in lymph and serum

The study of particulate fat in lymph and serum, even with the modern aids, still gives results which are difficult to interpret. This is because of the complex dynamics of the different lipid components and the different lipoprotein fractions.

The lipoproteins of serum are probably derived from three main sources. One is from thoracic duct lymph particles representing mainly exogenous lipid from the intestine, but also containing endogenous lipid synthesised in the intestinal wall (Lindsey and Wilson, 1965; Wilson and Reinke, 1968) and serum lipoproteins which reach the lymph by transudation from blood capillaries and liver (Courtice, 1968). The particulate fat in serum derived from the gut lumen and seen in abundance in chyle after a fatty meal have also been termed 'primary particles' by Bierman (1965).

A second source of serum lipid particles are probably derived mainly from the liver (Jones et al, 1966; Belfrage, 1968; Jeanrenaud, 1968) and may be seen in serum even after prolonged starvation. These particles are synthesised by the liver from free fatty acids or glucose depending to some extent on the animal species.

A third source is derived originally from primary particles but represents a breakdown or exchange of some of the lipid components of these particles. This alteration of particles may occur in the liver, in adipose tissue or at other sites and has been demonstrated in serum *in vitro* by the action of enzymes such as clearing factor lipase when the triglyceride component may undergo hydrolysis resulting in a smaller particle with relatively more cholesterol and phospholipids (Lindgren *et al*, 1955; Shore and Shore, 1962; Lossow *et al*, 1963).

Within the blood stream itself exchanges of the lipid components between chylomicrons, lipoproteins of different classes and red blood cells may occur. For example, an exchange of cholesterol and phospholipid has been described between lipoprotein fractions (Goodman, 1962; Roheim *et al*, 1963; Minari and Zilversmit, 1963; Zilversmit, 1968a and b). Adsorption of smaller particles on to larger particles also seems likely (Courtice and Garlick, 1962; Furman *et al*, 1962).

The source and fate of particulate lipid in the blood stream is therefore hard to interpret since the dynamics are complex, with particles reaching the blood stream from a number of different sources, losing

portions of lipids at different rates, being removed from the serum at different rates and at various sites and exchanging lipid between each other and other tissues. Thus, from a static sample of serum, even with the aid of morphology and lipid chemistry, it is very difficult to know what really is happening as regards the dynamics of the lipid exchanges at that time. An attempt will be made to rationalize some of these problems with particular reference to cholesterol in this thesis.

4. Aims and objects of this thesis

The introduction has dealt briefly with lipid absorption and transport and their possible association with lipid deposition in arterial walls. It has also explained some of the methods to be used in this study as well as giving a historic background leading to present ideas.

The study of lipid transport and its possible relationship to the development of experimental atherosclerosis in the rabbit is the overall object of this thesis.

The first group of experiments were designed to examine the morphology and makeup of lipid particles before entry into the blood stream. This involved the

collection of thoracic duct lymph from rabbits and the design of an electron microscopic method to measure the size distributions of the lipid complexes from different fractions of lymph separated by the ultracentrifuge. The comparison of the size of chylomicrons in the thoracic duct lymph after various dietary loads of triglyceride was also studied as was the size of lipid complexes carrying cholesterol before entry into the blood stream, since there has been evidence to suggest that the removal of cholesterol from the blood stream depends to some extent on the size of the lipid complex in which it is carried.

The knowledge of the morphology and lipid chemistry of thoracic duct lymph chylomicrons enabled a better assessment of their structure to be made and led to a clearer understanding of their synthesis in the mucosal cell.

Experiments were also designed to examine artificial fat emulsions since these have recently been used clinically for intravenous feeding and represent an artificial pathway for particulate lipid to enter the blood stream. It was decided to compare the sizes of these particles with those from the natural fat emulsion (chyle) and to see if a way could be suggested for making them more physiological.

A comparison between the equivalent lipoprotein fractions of serum and lymph in the cholesterol-fed rabbit was then examined and an explanation explored for the observed relative loss of triglyceride and gain in cholesterol of particles from serum as compared to lymph. An experiment was designed to examine the action of clearing factor lipase on lymph chylomicrons *in vitro* which resulted in a suggested explanation for the above phenomenon.

It was decided also to examine briefly the disappearance from the blood stream of lymph chylomicrons and very low density lipoproteins to see if particulate lipid of different sizes behaved differently.

Finally it was decided to design experiments with rabbits eating identical amounts of cholesterol but with different triglyceride loads of both corn oil and butter to see if their cholesterolaemia and the development of experimental atherosclerosis varied. It was found that those rabbits fed high doses of triglyceride with cholesterol did not develop hypercholesterolaemia or atherosclerosis to the same extent as those fed cholesterol alone.

As will be seen in the discussion many questions remain to be answered before any general hypothesis

arising from this work can be substantiated. Further possible experiments will be discussed. A theory, however, will be brought forward suggesting that the development of experimental atherosclerosis in the rabbit may to some extent depend on the size of the lipid particles carrying cholesterol from the thoracic duct lymph to the blood stream, which in turn depends on the amount of fat eaten with the cholesterol in the diet.

MATERIALS AND METHODS

1. Experimental Animals

Rabbits were a cross New Zealand White and Californian breed of both sexes. After weaning at about six weeks of age they were housed in individual metabolism cages with wire bases and fed powdered rabbit pellets in dishes with rims to prevent the spillage of food.

Rabbits aged about 15 weeks and weighing 2-3 kg were fed added triglyceride in the earlier experiments, but in later experiments where it was essential for the rabbits to eat their full diet from the first day of offering, as when serial serum cholesterol levels were compared, rabbits of only 8-10 weeks of age and 1.5 - 2.0 kg weight were used.

Young rats of about 150 g weight of the Wistar strain were also used in some experiments.

2. Components of the different diets fed to rabbits

(i) Powdered pellets

The basic component of the diet was powdered rabbit pellets supplied by Drug Houses of Australia, Rural Division, Alexandria, N.S.W. The pellets were composed of Soya Bean Meal, Linseed Meal, Lucerne Meal, Wheat Meal, Wheat Germ, Wheat Offal (comprised of flour, bran, pollard, wheat germ and other offals,) Pollard, Yeast, Salt, Boneflour, Magnesium Oxide and Vitamin C, with the following approximate chemical composition:-

Digest. Protein	17.5%	Niacin	10.0 mg/100 g
Crude Protein	20.0%	Pantothenic Acid	2.0 " " "
Crude Fat	5.0%	Folic Acid	0.5 " " "
Crude Fibre	10.0%	Choline	100.0 " " "
Salt	1.0%	Betaine	1.0 " " "
Calcium	1.2%	Vitamin K	+ " " "
Phosphorous	0.6%	E	6.0 " " "
Manganese	0.005%	C	35.0 " " "
Copper	0.001%	Biotin	+ " " "
Potassium	0.5%	Methionine	0.2%
Iron	0.02%	Lysine	1.2%
Magnesium	0.4%	Argentine	0.7%

Vitamins A	500 iu/100 g	Cystine	0.3%
D3	4 iu/100 g	Tryptophan	0.3%
B1	1.6 mg/100 g	Glutamic Acid	3.0%
B2	1.5 " " "	Histidine	1.4%
B6	1.6 " " "	Leucine	6.0%
B12	+ " " "	Phenylalanine	0.7%
		Valine	0.7%

(ii) <u>The lipid composition of powdered pellets and the preparation of a lipid free powdered pellets diet</u>.

One group of rabbits was fed lipid free food. This was prepared by a 24 hr continuous ether extraction of the pellets in a large Soxhlet apparatus capable of holding 1 kg of food at a time. From an original 4000 g of powdered pellets 3,700 g of ether extracted pellets were obtained. Vitamins D and E were added again to this extracted food in original concentrations. The weight of the ether soluble extract, after the removal of water by drying in a Bucchi vacuum evaporator with chloroform-methanol, was 124 g.

From this data it appears that the original powdered pellets contained about 3 per cent ether soluble lipid and approximately 4 per cent water. The sterol content of the food, measured as cholesterol by

the colorimetric method of Zak et al (1954) after saponification (Abell et al 1952) was 104 mg/100 g of food or about 0.1 per cent. This probably was mainly phytosterol. This result compares with that of Aramaki et al (1967) who found 113 mg of Liebermann-Burchard positive substances, calculated as cholesterol, in 100 g of Japanese rabbit chow.

(iii) The lipids added to the diet

(a) Cholesterol The cholesterol fed to rabbits was 'pure cholesterol' supplied by Townsend and Mercer. On analysis compared with highly purified ash free cholesterol (Bacto-cholesterol, Difco Laboratories, Michigan, U.S.) the cholesterol was 85 per cent pure.

(b) Corn Oil The corn oil used in the diets was 'Royles Kentucky Corn Oil'. The sterol content measured as cholesterol was 808 mg/100 ml or 878 mg/100 g which compares with the figure in Cook (1958) of 800 mg/100 g . The sterol of corn oil is made up of 65 per cent β-sitosterol, 30 per cent campesterol and 5 per cent stigmasterol (Grundy et al 1968).

(c) Soya bean oil 'Vales Pure Soya Bean Oil' was fed to rabbits in some experiments. It contained 300 mg/100 g of sterol measured as cholesterol,

in comparison to Cook's figure of 200 mg/100g. This again was probably mainly β-sitosterol.

(d) Butter The butter used to feed the rabbits was 'Foley's' butter, blended by the Producers' Co-operative Distributing Society Ltd., Sydney. The composition of the butter as supplied by the chief chemist of the manufacturers, was as follows:-

Butter fat	80%
Moisture	16%
Salt, NaCl	2%
Curd comprising mostly protein	2%

The sterol content of the butter, measured as cholesterol, was 200 mg/100 g and was probably cholesterol because of its animal origin.

(e) Frymasta This vegetable shortening or hard fat was manufactured by Frymasta Vegetable Oils Pty. Ltd. of Australia and contained primarily palmitate and oleate as fatty acids (Zilversmit et al 1967). The sterol content was not determined.

(iv) Blending of the different dietary mixtures fed to rabbits

The basic component of all the diet mixtures was powdered pellets which will subsequently be referred to as 'plain food'. The various fats and cholesterol as

described above were added to the plain food on a weight for weight basis, and thoroughly mixed. The different diets in addition to plain food alone were:-

(a) 0.8 per cent cholesterol in plain food

(b) 0.8 per cent cholesterol in ether extracted plain food.

(c) 0.8 per cent cholesterol with 5, 15 or 30 per cent corn oil in plain food.

(d) 0.8 per cent cholesterol with 5, 15, 30 or 37 per cent butter in plain food.

(e) 0.8 per cent cholesterol with 30 per cent soya bean oil in plain food.

(f) 0.8 per cent cholesterol with 30 per cent Frymasta in plain food.

(g) 5, 10 or 30 per cent corn oil in plain food without added cholesterol.

(h) 5, 10 or 30 per cent butter in plain food without added cholesterol.

(v) Calorie content of the various diets

Exactly 50 g of one of the above food mixtures was fed to the rabbits each morning. With dry mixtures some water was mixed after weighing and before feeding to render the powder more palatable. The total calories fed per day were made approximately equal in all cases

by feeding the rabbits on the lower triglyceride diets a calculated extra amount of plain powdered pellets.

(vi) Sterol content of the diets

In the cholesterol feeding experiments although the daily intake of added cholesterol was 400mg in all cases, the daily total sterol intake varied. For example, those rabbits eating 69g of plain food only received about an extra 70mg of sterol (phytosterol) from the pellets. Those animals eating 30 per cent corn oil, however, received about an extra 165mg per day whereas those animals eating 30 per cent soya bean oil received an extra 80 mg per day. Rabbits eating 30 per cent butter received an extra 65 mg sterol per day, about 30 mg of which was cholesterol from the butter and about 35 mg phytosterol from the food.

The possible significance of the extra sterol content of the different diets fed to the cholesterol fed rabbits will be discussed later.

3. Collection and preparation of biological samples

(i) Blood

Blood was collected from the marginal vein of the ear of unanaesthetised rabbits. When large quantities of blood were required as for the clearing

factor experiments or for ultracentrifugal separation of lipoprotein in serum, it was collected from a cannula in the carotid artery or by puncture of the central artery of the ear.

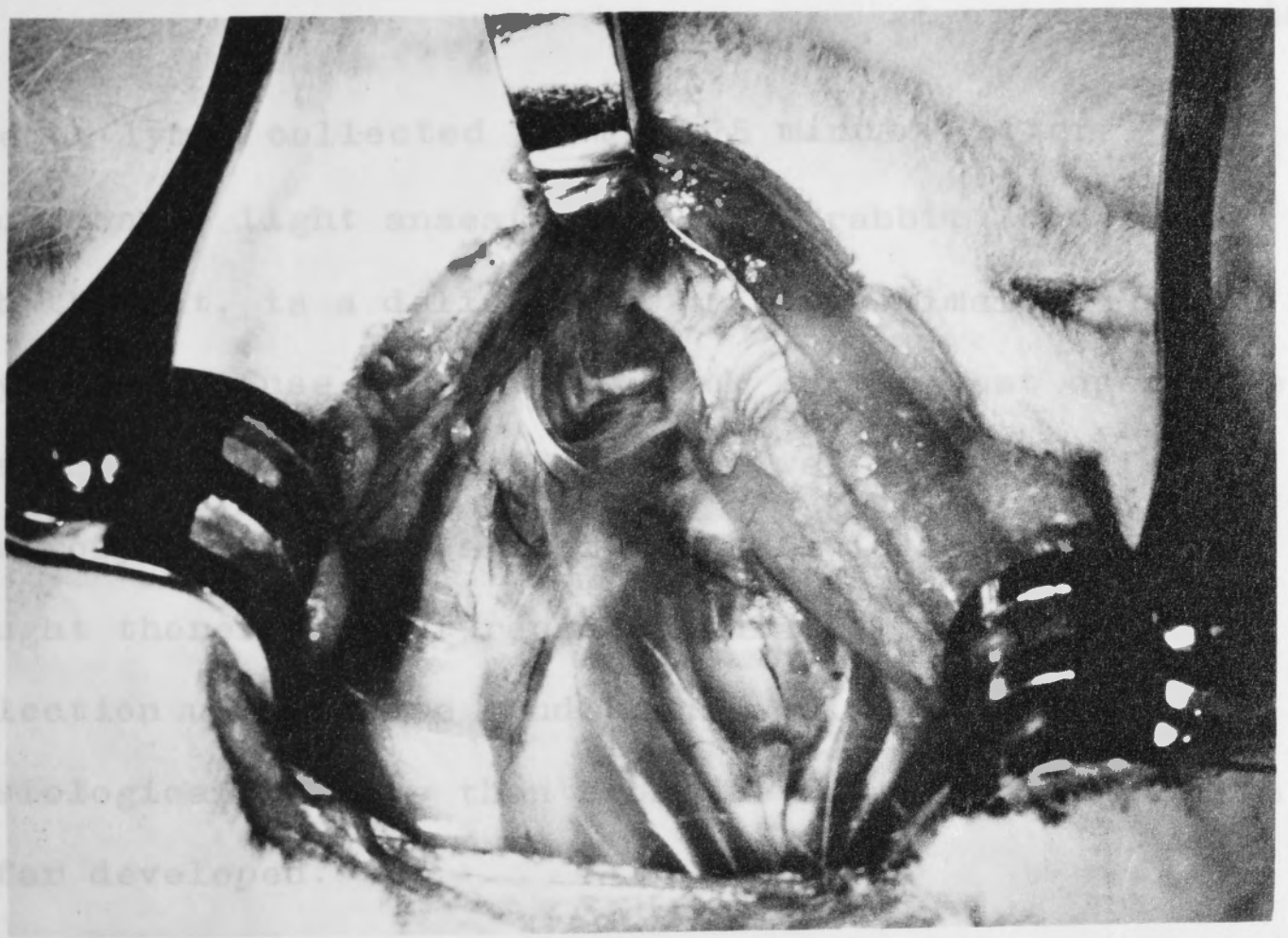
(ii) Thoracic duct lymph

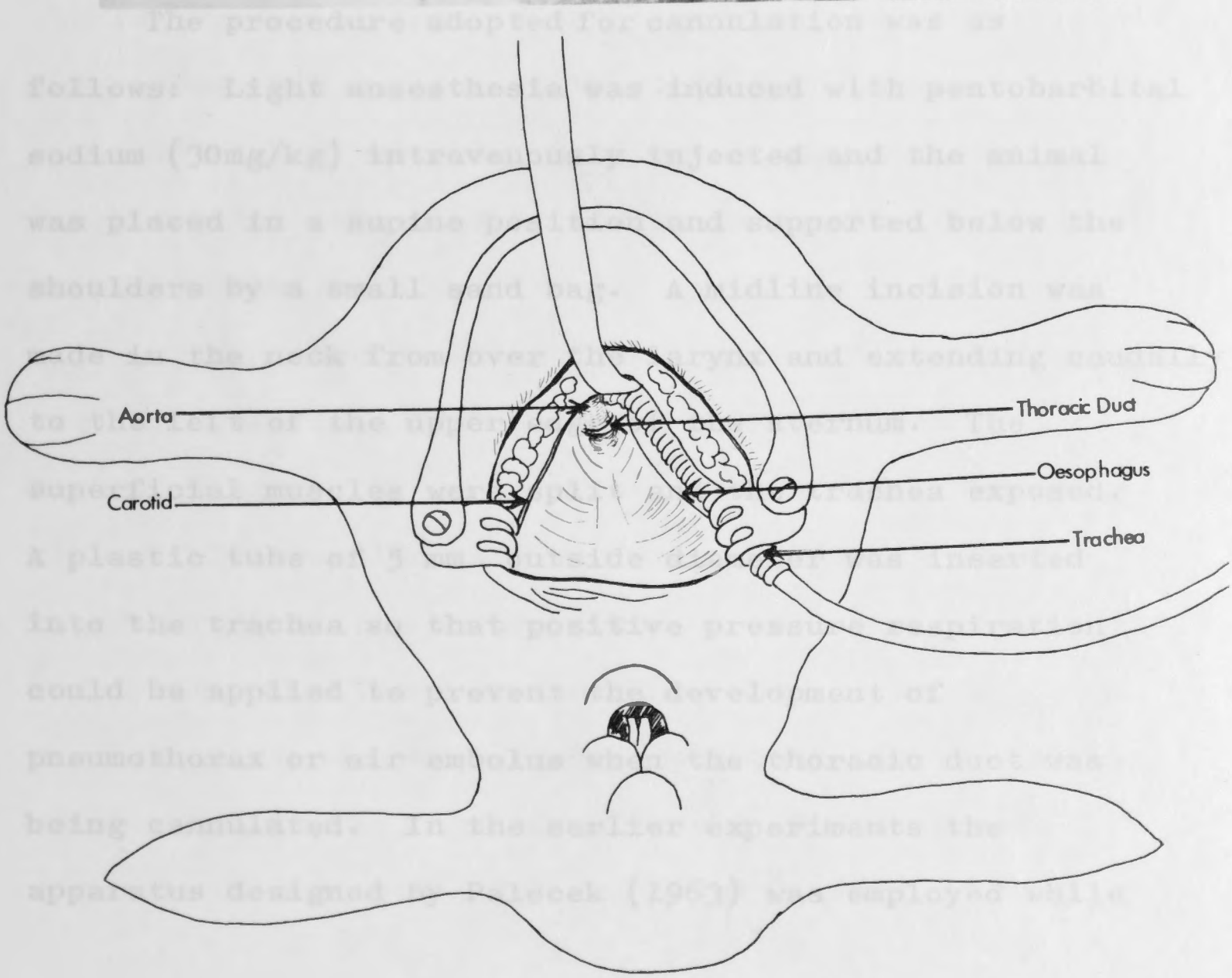
Thoracic duct lymph from rats was collected by the method of Bollman et al (1948). In the rabbit however the duct was cannulated in the apex of the thorax by the technique developed under the guidance of Dr Zilversmit during his visit to Canberra in 1966 (Zilversmit et al 1967). The surgical approach as illustrated in Fig. 1. makes the location of the thoracic duct relatively easy especially if the rabbits have been previously fed triglyceride to colour the duct white. The duct in this position is constant, (no variant or branching has been observed in any rabbit so far cannulated) straight and has fewer valves than near its entry into the veins at the root of the neck. In the occasional cases where cannulation has failed lymph can still be collected by means of a pipette since the tissue planes formed make an ideal bloodless collecting funnel.

Although it is possible to prepare a chronic preparation by this method most of the experiments

Figure 1

Schematic diagram and photograph of dissection to expose the thoracic duct.




Aorta
Carotid
Thoracic Duct
Oesophagus
Trachea

were on lymph collected from 15-75 minutes after induction of light anaesthesia. The rabbit, compared with the rat, is a delicate laboratory animal and in many cases refuses to eat or drink in the post operative phase. The animals therefore lose weight and suffer electrolyte disturbance from lymph fluid loss. It was thought therefore that rapid cannulation and lymph collection under acute conditions would give more physiological results than from the chronic preparations so far developed.

The procedure adopted for cannulation was as follows: Light anaesthesia was induced with pentobarbital sodium (30mg/kg) intravenously injected and the animal was placed in a supine position and supported below the shoulders by a small sand bag. A midline incision was made in the neck from over the larynx and extending caudally to the left of the upper edge of the sternum. The superficial muscles were split and the trachea exposed. A plastic tube of 5 mm outside diameter was inserted into the trachea so that positive pressure respiration could be applied to prevent the development of pneumothorax or air embolus when the thoracic duct was being cannulated. In the earlier experiments the apparatus designed by Palecek (1963) was employed while

later a Bird (Bird Corporation, Palm Springs, California) respirator Mark 7 or 8 was used to provide oxygen at intermittent pressures of 10 to 20 cm of water.

The left sternomastoid and pectoralis major muscles were partially severed near their origin from the sternum and the trachea and esophagus separated from the left carotid sheath along tissue planes by insertion of a Mayo's retractor. By this means the trachea, esophagus and right common carotid sheath were displaced to the right, and left carotid sheath, the left external jugular vein and the left sternomastoid and sternohyoid muscles to the left. The sternum was elevated with a retractor held by an assistant, or more conveniently, connected by string to a pulley with a hanging weight.

This procedure exposed the upper mediastinum on the left side in a funnel shaped cavity formed by tissue planes. By means of a glass rod the connective tissue in the mediastinum was removed down to the upper margin of the aortic arch, at the level of the first rib. The thoracic duct then usually became visible as it passed on the right dorsal side of, and in close proximity to, the aortic arch. The duct was ligated and a second ligature placed loosely around

it a few millimeters caudally. Polyethylene or polyvinyl tubing (Dural Plastics and Engineering Pty, Dural, N.S.W.) of 0.5 mm internal diameter and 0.15 mm wall thickness was inserted into the duct by passing it through a 17 gauge needle which was used to puncture the duct. Sometimes the duct was cut with scissors and the catheter inserted, but this was more difficult particularly in animals producing a very milky lymph which on escape obscured the hole in the duct. The plastic tubing could frequently be inserted several centimeters into the duct but on occasions was stopped by a valve only a millimeter or so from the point of insertion. In acute experiments one hour's flow of lymph was collected directly into a centrifuge tube and the rabbit then destroyed.

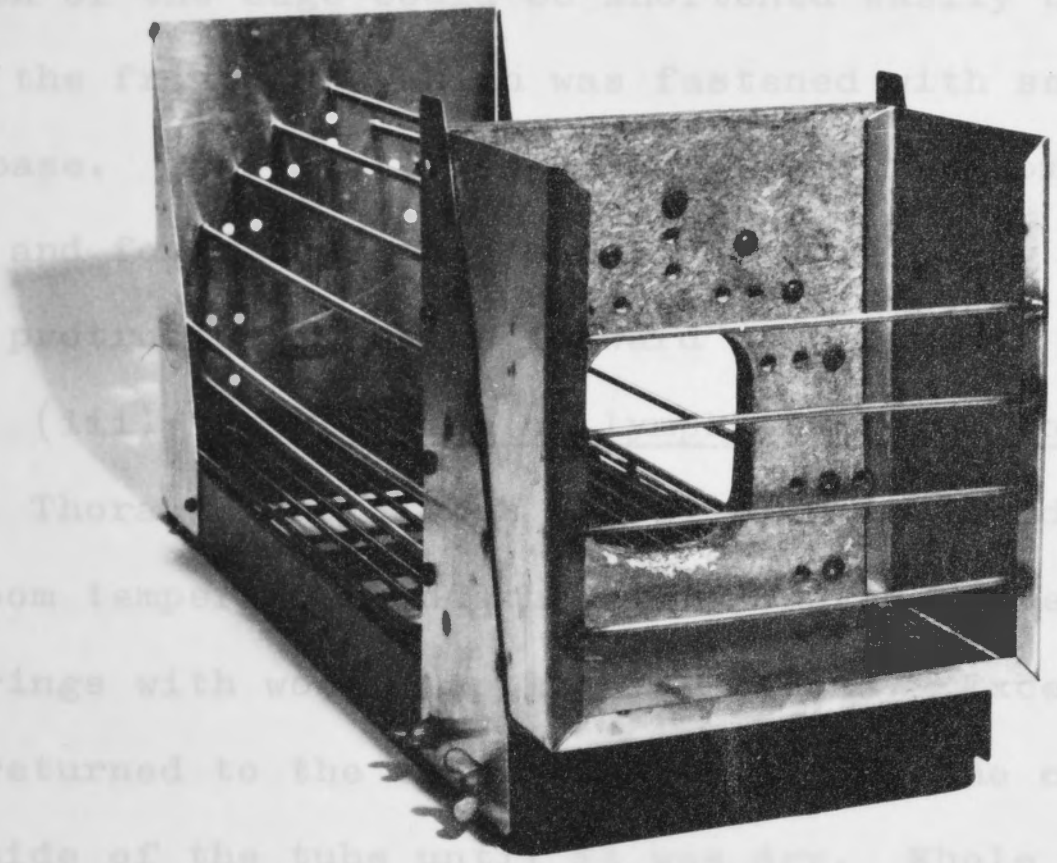
In chronic experiments the trachea was intubated 'blind' through the mouth, and after cannulation the cannula was passed under the skin to a suitable exit anywhere along the back or side of the animal. The wound was closed with sutures, the tracheal tube removed and penicillin administered intermittently for 48 hours. After the operation the rabbit was transferred to a restraining cage (Fig. 2). The cages were 18 inches long, 6.5 inches wide and 9 inches high. The effective

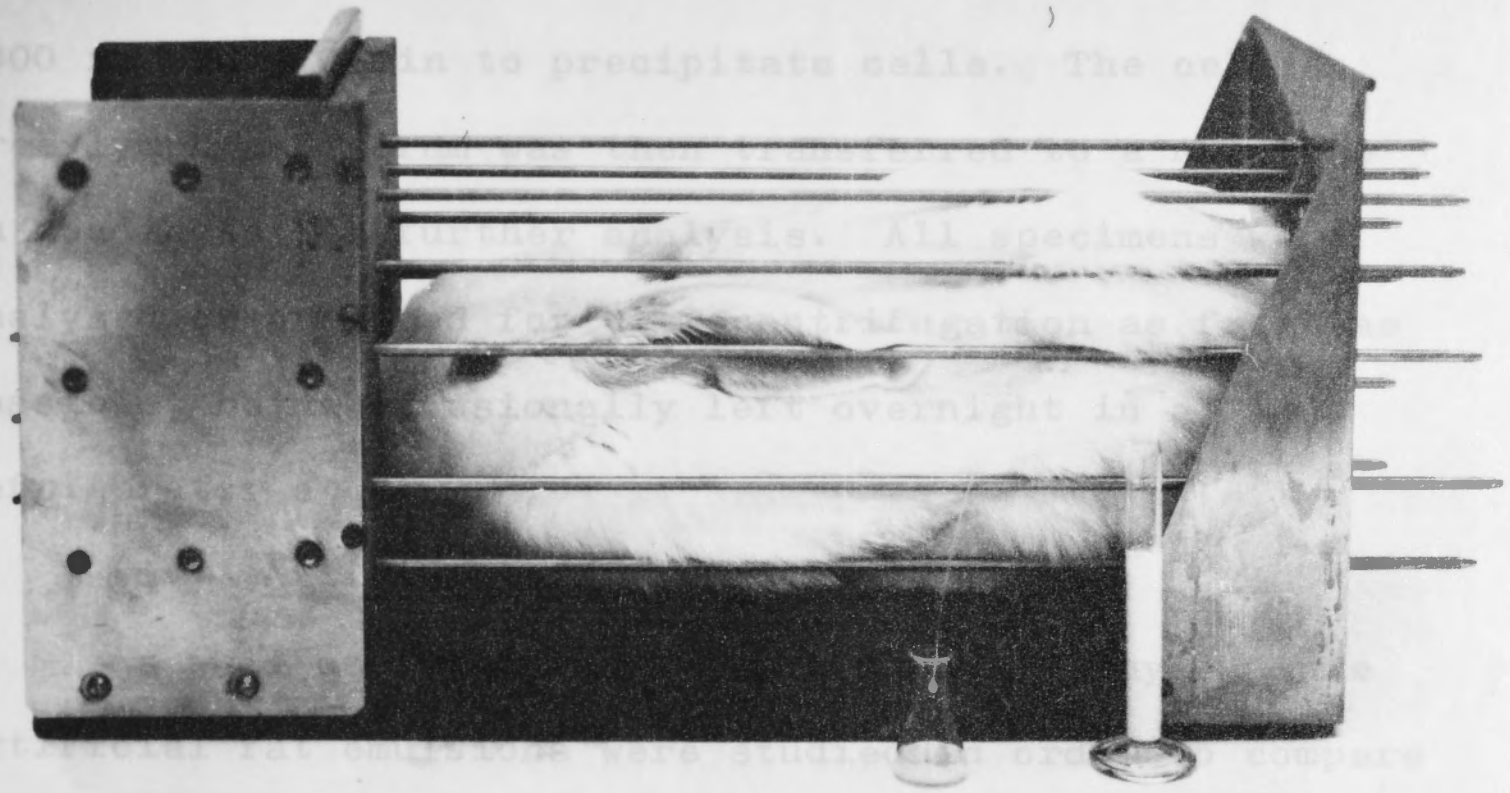
Figure 2

Restraining cage for rabbits.

Top: Front compartment, for food and water, does not show the metal slide holding the restraining bars in place.

Bottom: Side view, metal slide in place





length of the cage could be shortened easily by sliding back the front end, which was fastened with screws to the base. The animal was allowed some freedom to move back and forth and could eat and drink freely with its head protruding into the forward compartment.

(iii) Preparation of lymph and serum for analysis

Thoracic duct lymph was allowed to stand for 1 hr at room temperature and the clot removed by repeated stirrings with wooden applicator sticks. Excess lymph was returned to the specimen by pressing the clot against the side of the tube until it was dry. Whole blood was allowed to clot at room temperature and the clot gently loosened from the side of the centrifuge tube. The samples were then spun in an ordinary centrifuge at 2000 rpm for 10 min to precipitate cells. The cell free lymph and serum was then transferred to a new tube in readiness for further analysis. All specimens were analysed or prepared for ultracentrifugation as fresh as possible, being occasionally left overnight in a refrigerator at 4^{o} C.

(iv) Artificial fat emulsions

As well as the natural fat emulsions (chyle) some artificial fat emulsions were studied in order to compare their morphology and chemical composition.

Lipomul manufactured by the Upjohn Co. Kalamazoo, Michigan, U.S.A. was one specimen studied. It consisted of 15 per cent triglyceride obtained from a cottonseed oil source emulsified with 1.2 per cent phospholipid from a soya bean source. Soya bean oil emulsions made by Vitram AB of Sweden were the most commonly examined emulsions. The commercial brand is known as Intralipid (I.L.) with a composition of either 10 or 20 per cent triglyceride from soya bean oil, emulsified with 1.2 per cent phospholipid from egg yolk.

Intralipids of batch numbers 193031 and 296024 will be known as 'old Intralipid' since they were manufactured in September 1963 and March 1966 respectively and examined in April 1968. Intralipids of batch numbers 197119 and 298036 will be known as 'new Intralipids' since they were manufactured in June 1967 and March 1968 and examined in August 1968. The pH of manufacture varied, the older Intralipids being manufactured at a relatively lower pH.

Experimental soya bean oil emulsions (SBO) were also supplied by the manufacturer. They were made by the same process and from the same sources of triglyceride and phospholipid as Intralipid. The concentrations of triglyceride and of phospholipid and the pH at manufacture

were, however, varied. Batch 140967 was one year old when examined but batch 150868 was only two weeks old.

The processing of I.L. and SBO consists of dissolving phospholipids in soya bean oil and glycerol in water. The two phases - oil and water - are then mixed under vigorous agitation to form a crude emulsion. The emulsion is then homogenized several times under regulated temperatures and pressures, the latter at some stages becoming very high. All phosphorus in the emulsion originates from egg yolk phospholipids and nothing is added except glycerol, water, oil and phospholipids. The soya bean oil emulsions contain, however, about 0.1 per cent tocopherols of natural origin (Håkansson, 1968, personal communication).

The formula for Intralipid as stated on the label was fractionated soya bean oil, 50 or 100 g, fractionated egg lecithin 6 g, dilute glycerol (Ph.Int.) 12.5 g, water for injection to 500 ml.

The triglyceride and phospholipid composition and the pH at manufacture are listed in Table I.

TABLE 1

THE TRIGLYCERIDE AND PHOSPHOLIPID CONTENT AND pH AT MANUFACTURE OF VARIOUS ARTIFICIAL FAT EMULSIONS

SBO Batch No. 140967	TG mg/100 ml	PL mg/100 ml	pH
A	20,000	150	6.7
B	20,000	300	6.5
C	20,000	600	6.9
D	20,000	1,200	6.7
E	20,000	1,200	5.8
F	20,000	2,400	6.3
G	20,000	4,800	6.6
SBO Batch No. 150868			
C	20,000	600	6.1
D	20,000	1,200	6.7
E	20,000	1,200	6.2
H	20,000	1,200	8.1
G	20,000	4,800	6.2
I	5,000	600	6.0
K	5,000	600	6.5
Intralipid			
I.L. 10% (old)	10,000	1,200	5.9
I.L. 10% (new)	10,000	1,200	8.1
I.L. 20% (old)	20,000	1,200	7.2
I.L. 20% (new)	20,000	1,200	7.8

4. Ultracentrifugal methods for the separation of lipoproteins in lymph and blood serum

The method of fractionation of lipoproteins by ultracentrifugation as used in this thesis was briefly described in a paper by Zilversmit et al (1967). The theory behind the method has been mentioned in the introduction.

(i) Separation of chylomicrons of Sf>400.(Dole & Hamlin 1962).

Whole lymph or serum was centrifuged in a Spinco SW 25.1 swinging bucket head if large quantities (30 ml x 3) were required for analysis, or in a SW 39L head for smaller quantities (5 ml x 3). The centrifuge used was a Spinco Ultracentrifuge model L (Beckman-Spinco Division, Palo Alto, California U.S.A.) and specimens were refrigerated to 7-10^{o} C before and during centrifugation.

The SW 25.1 head was first spun for 1 hr at an average centrifugal force of 64,000 x G. resulting in a tightly packed creamy layer of chylomicrons at the top of the tube. The subnatant was carefully removed by inserting a 22 gauge needle through the chylomicron layer to the bottom of the tube and aspirating about 80 per cent of it into a syringe. The remaining creamy

chylomicron layer and some of the subnatant layer were resuspended in a salt solution of density of 1.2 g/ml and over this a 5 cm layer of saline of density of 1.006 g/ml was gently layered. The sample was for a second time centrifuged at 64,000 x G. for 2 hr to wash the chylomicrons up through the salt gradient leaving behind smaller particles. Again, a top creamy layer resulted henceforth called chylomicrons of $S_{f>400}$ (see nomogram in Dole and Hamlin 1962), which was separated by means of a tube slicer for further analysis or for electron microscopy.

A similar procedure was followed for the smaller but faster spinning SW 39L head but the times and forces of centrifugation were 0.5 hr and 1 hr at 125,000 x G. average centrifugal force.

(ii) <u>Separation of VLDL of Sf 12-400 and of lipoproteins of density>1.019</u> (Havel et al 1955).

The subnatant obtained after the first centrifugation in the preparation of the chylomicron fraction contained all the lipoproteins except chylomicrons ($S_{f>400}$). This chylomicron free sample was adjusted to density of 1.019 g/ml by the addition of 1 volume of salt solution of density 1.085 g/ml to 5 volumes of sample (density

1.006 g/ml). The sample was then centrifuged in a Spinco 40.3 or 50 angle rotor for + 16 hr at 100,000 x G. or more and then separated into an upper and lower fraction by slicing. Lipoproteins of density less than 1.019 g/ml (known as VLDL) were thus in the upper fraction and greater than 1.019 g/ml (known as D>1.019) in the lower fraction.

On some occasions for electron microscopic purposes the D>1.019 fraction was further adjusted to a density of 1.21 g/ml and spun for a further 30 hr at +100,000 x G. to separate the plasma proteins from the lipoproteins.

5. <u>The nomenclature used in this thesis for the lipoprotein fractions as separated by the ultracentrifuge</u>.

The fractions separated by these methods depicted in Fig. 3 are:-

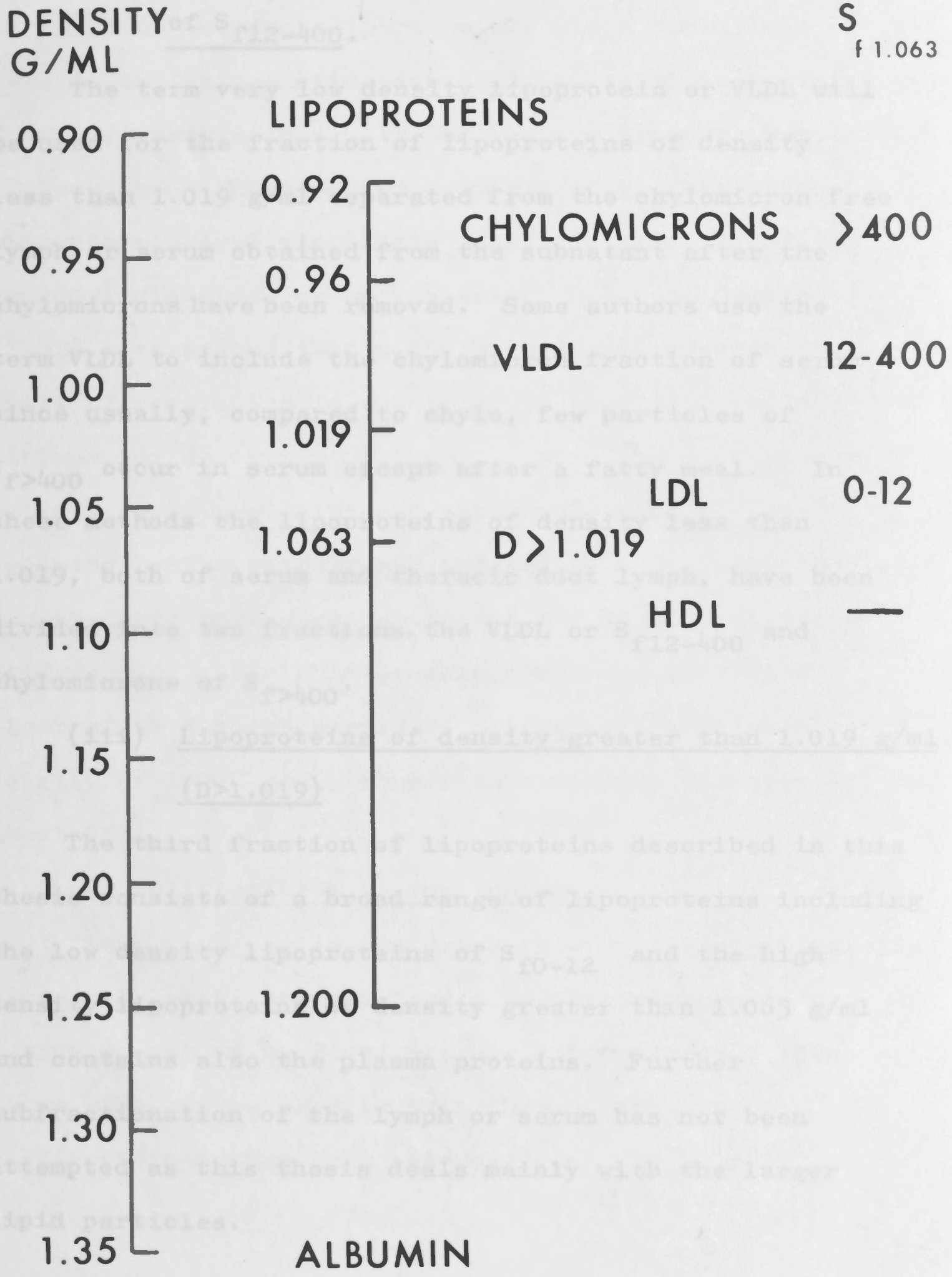
(i) <u>The chylomicron fraction ($S_{f>400}$)</u>

The term chylomicrons ($S_{f>400}$) will be used for all particulate lipid whether derived from lymph or serum by the first procedure and spun through the saline gradient in the swinging bucket rotor.

Figure 3

A schematic diagram of the lipoprotein fractions as separated by ultracentrifugation methods used in this thesis

METHOD OF FRACTIONATION



(ii) Very low density lipoprotein fraction (VLDL) of $S_{f12-400}$.

The term very low density lipoprotein or VLDL will be used for the fraction of lipoproteins of density less than 1.019 g/ml separated from the chylomicron free lymph or serum obtained from the subnatant after the chylomicrons have been removed. Some authors use the term VLDL to include the chylomicron fraction of serum since usually, compared to chyle, few particles of $S_{f>400}$ occur in serum except after a fatty meal. In these methods the lipoproteins of density less than 1.019, both of serum and thoracic duct lymph, have been divided into two fractions, the VLDL or $S_{f12-400}$ and chylomicrons of $S_{f>400}$.

(iii) Lipoproteins of density greater than 1.019 g/ml (D>1.019)

The third fraction of lipoproteins described in this thesis consists of a broad range of lipoproteins including the low density lipoproteins of S_{f0-12} and the high density lipoproteins of density greater than 1.063 g/ml and contains also the plasma proteins. Further subfractionation of the lymph or serum has not been attempted as this thesis deals mainly with the larger lipid particles.

(iv) Particulate lipid

The definition of particulate lipid probably depends on the magnification used for resolving particles. In early observations with the light microscope, particles of less than 0.5μ were hard to distinguish whereas now with the electron microscope small lipoproteins of 100 or 200 Å diameter can be resolved. Dole and Hamlin (1962) have stated that with the light microscope the limit of resolution is about 200 mμ ranging down to a minimum for detection of about 75 mμ. The distinction between small particles and large molecules is thus becoming both arbitrary and indefinite. Bierman (1965), has used the term 'fat particles' for light scattering triglyceride rich lipid-protein complexes that will float after a short period of ultracentrifugation at a density of 1.006 g/ml, thus with a minimum diameter of about 750Å.

(v) Difficulties in terminology

There might arise confusion from various terminologies especially when terms derived from electrophoresis are included such as α, β and pre - β lipoproteins. These terms will not be used in this thesis since no electrophoresis has been attempted.

Until more is known about the source, morphology, dynamics and final destination of the lipoproteins the terminology will remain confusing. The chylomicron fraction of thoracic duct lymph probably represents the most pure fraction since most of the lipid particles in this fraction are derived from the same source, the mucosal cell of the small intestine. Most lipoprotein fractions, however, whether separated by ultracentrifugation, electrophoresis or immunological methods, do not represent pure species of particles, but a complex spectrum of particles from many varied sources, undergoing complex exchanges.

6. The use of the electron microscope to determine the size of lipoproteins

A method was devised to measure the diameter distribution, surface area and volume of chylomicrons from electron micrographs.

(i) Preparation of specimens

The fixation and visualization of chylomicrons and lipoproteins was similar to the methods used by Hayes and Hewitt (1957) and Bierman et al (1966). One or more drops of whole chyle or of the reconstituted chylomicron or lipoprotein fractions were added to 1 per cent osmium tetroxide solution. The solution became

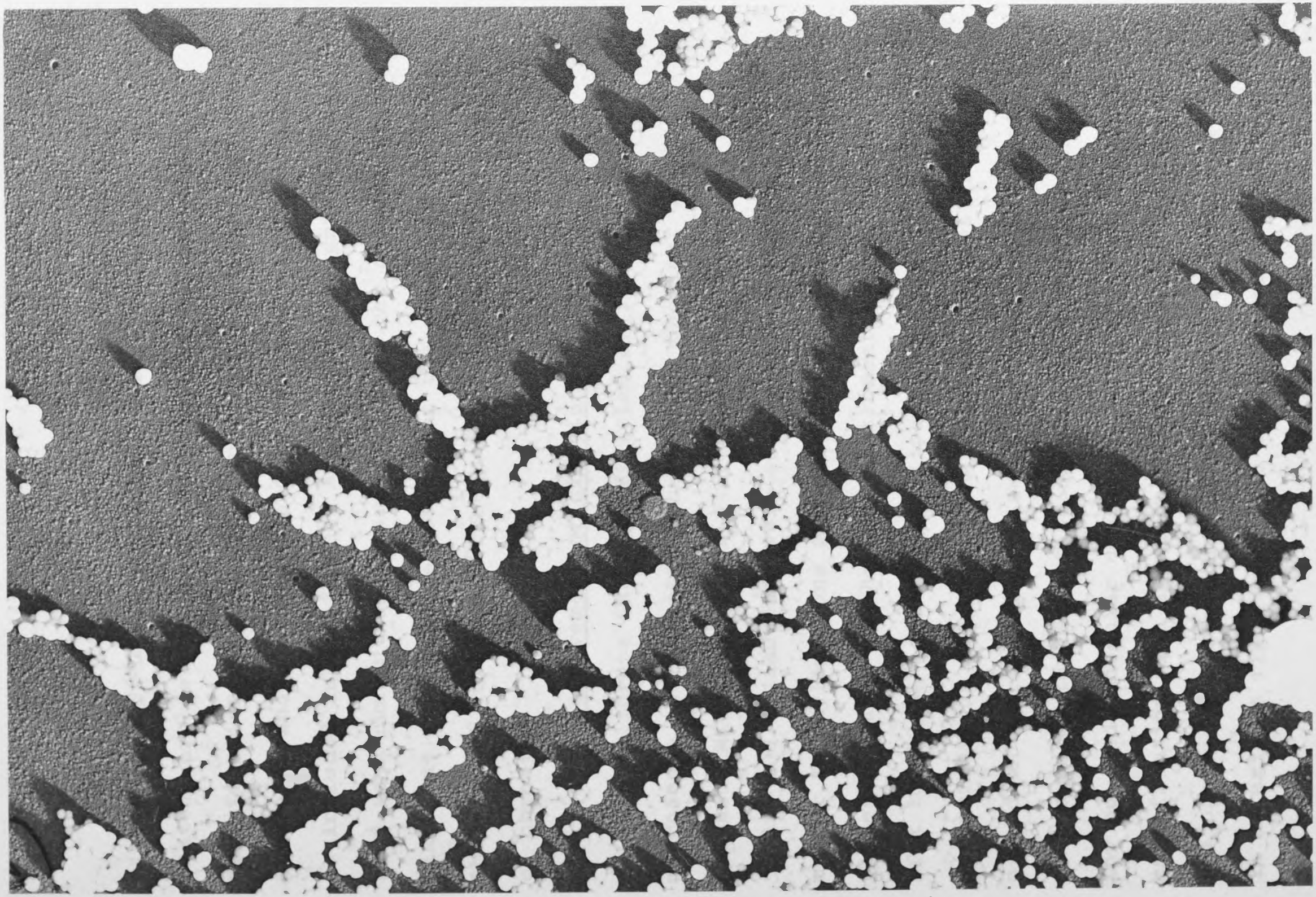
a light brown colour and contained about 1 mg/ml of triglyceride. This concentration gave an osmium to triglyceride ratio higher than the highest concentrations used by Kay and Robinson (1962) in order to ensure adequate fixation.

The suspension was allowed to stand for 1 hour at room temperature, was then thoroughly mixed to resuspend the fixed lipid particles which tended to precipitate, and a drop of the suspension was placed on a carbon coated grid. Excess fluid was blotted off the grids before processing in a vacuum evaporator (Type JEE 4B, Japan Electron Optics Laboratory Co, Ltd). After a vacuum of 6×10^{-5} mm mercury pressure was produced chromium was evaporated from a point source at a distance of not less than 15 cm from the specimen in order to keep the penumbra minimal (Kay 1961) and at an angle of about 15 degrees to throw long shadows. The grids were then examined in a Siemens Elmiskop 1 electron microscope and random fields photographed at a constant instrumental magnification of x 10,000.

As can be seen from Fig. 4, many chylomicrons are in contact with each other indicating that no evaporation or shrinkage of lipid has occurred after they were first placed on the grid or during vacuum procedures. Electron

Figure 4

Electron micrograph of chylomicrons from the thoracic duct of a corn oil fed rabbit showing the shadows cast by chromium and indicating their spherical shape and variation in diameters



micrographs of lipid particles, unless otherwise stated, are reverse positive prints at an approximate magnification of x 30,000. In most cases a polystyrene sphere of known diameter and at the same magnification is included as a reference scale. Reverse printing is used as a convention in order to make the shadows appear black but it should be noted that the shadow is actually the area of carbon membrane not covered by a fine layer of chromium.

(ii) Measurement of diameters

To calibrate the magnification of the electron microscope, polystyrene spheres (Dow Chemical Co., Midland, Michigan, U.S.A.) of stated 880 $\pm$ 80 and 5570 $\pm$ 100 $\mathring{A}$ diameters were shadowed in a similar manner to chylomicrons for electron microscopy and plates prepared of the same instrumental magnification of x 10,000 as used for all lipoprotein size determinations (Fig. 5). The resultant plates were placed on a transparent plastic holder which was divided into squares to facilitate counting and examined under a low powered binocular dissecting microscope at constant magnification with a calibrated micrometer scale in one eye piece. The diameters of all particles were measured in an axis at right angles to their shadows (Misra and

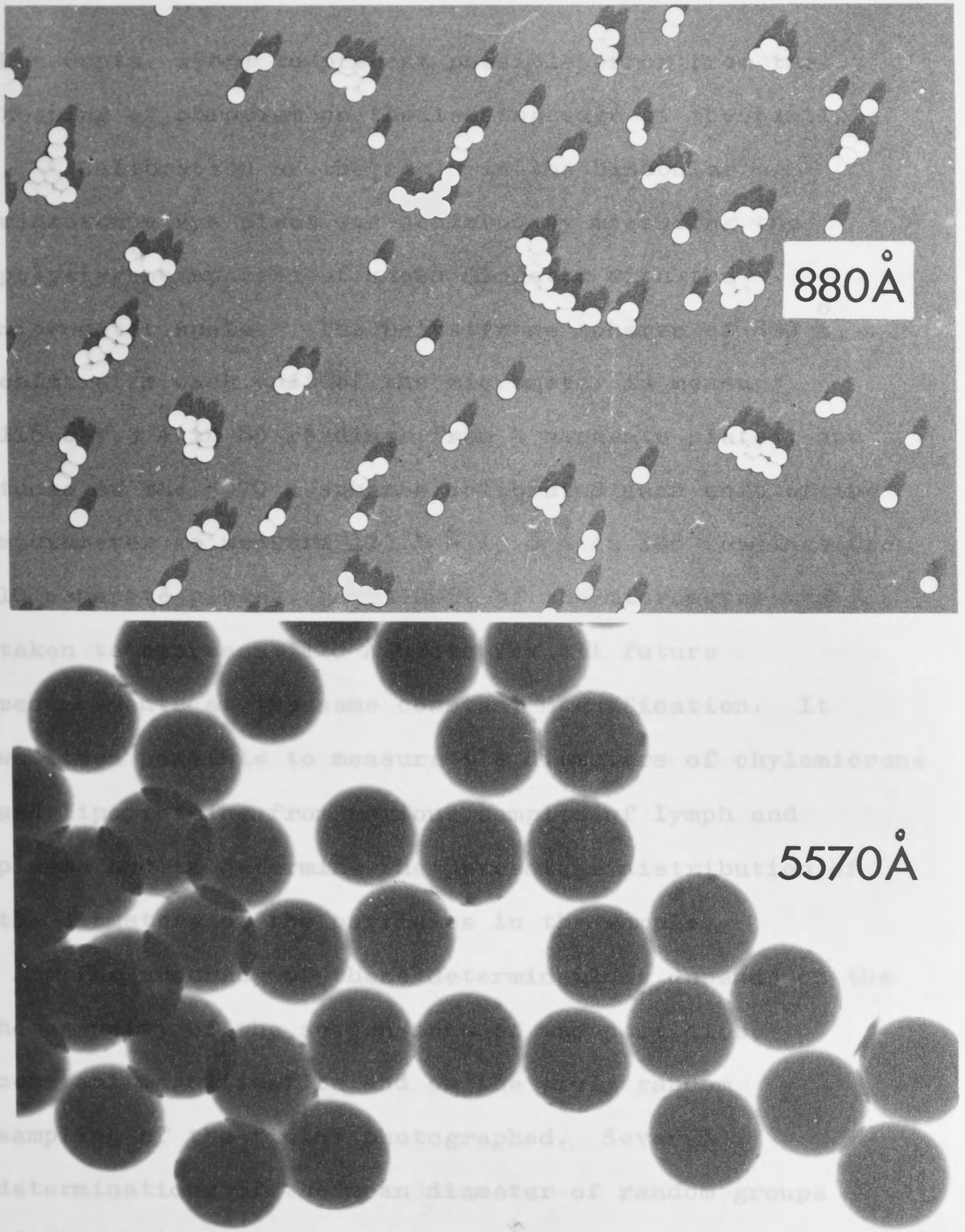
Figure 5

Electron micrographs of polystyrene spheres used for calibration purposes.

(1) Upper picture shows shadowed spheres of 880 Å diameter

(2) Lower picture shows unshadowed spheres of 5570 Å diameter.

POLYSTYRENE SPHERES



Das Gupta, 1965) to prevent possible error from the heaping of chromium on the leading edge of the particle.

Calibration of the scale in the binocular microscope eye piece was achieved by measuring the polystyrene spheres of known diameter with the micrometer scale. The polystyrene spheres of 880 $\mathring{A}$ calibrated each unit of the micrometer to measure 116.6 ± 7.3 $\mathring{A}$ in 80 readings from 4 separate plates, and those of the 5570 $\mathring{A}$ spheres calibrated each unit of the micrometer to measure 123.8 ± 13.6 $\mathring{A}$ in 108 readings from 10 separate plates. Each unit of the micrometer was taken to represent 120 $\mathring{A}$ units for all future measurements at the same constant magnification. It was then possible to measure the diameters of chylomicrons and lipoproteins from various samples of lymph and plasma and to determine the percentage distribution of the diameters of the particles in the sample.

The accuracy of these determinations depends on the homogeneity of the suspensions of the particles, constant magnification and on the truly random sampling of the fields photographed. Several determinations of the mean diameter of random groups of 200 chylomicrons were made from the same sample of chylomicrons to test for reproducibility. The average

of 8 such determinations on chylomicrons from thoracic duct lymph of a rabbit eating 30 per cent corn oil was 1598 ± 48 Å, the count being made from 12 plates obtained from 4 grids. The average of 6 determinations from an animal eating 5 per cent corn oil was 998 ± 31 Å, the counts being made from 6 plates obtained from 3 grids. These figures suggest that the suspensions were homogeneous and the samples truly random.

The possibility that ultracentrifugation might cause coalescence of chylomicrons resulting in larger agglomerates was also checked by treating whole chyle in the same way as the chylomicron fractions. The average of the determination of 8 means of chylomicron diameters from fresh whole lymphs (particles of diameter less than 720 Å were not counted) of the rabbit eating 30 per cent corn oil was 1569 ± 42 Å. That a similar range of chylomicron sizes was observed suggested that ultracentrifugation and resuspension of chylomicrons did not cause agglomerates.

(iii) Calculation of the mean surface area and mean volume of a random sample of particles

Lipid particles from the thoracic duct lymph of rabbits or rats absorbing corn oil have been shown by electron microscopy to be spherical in shape. The

diameters (d) of a homogenous random sample of 100 or 200 particles are first determined. Since the surface area (SA) of a sphere is equal to πd^2 and the volume (V) of a sphere is $\frac{\pi d^3}{6}$ the surface area and volume of each individual particle can be calculated. This was done with reference to Table 2 in which, for convenience, the areas and volumes of some spheres of different diameters have been tabulated. The total surface area (Σ SA) and total volume (ΣV) of 100 or 200 particles were then determined by addition of the individual SA and V. From these sums the mean surface area $\overline{SA}$ and mean volume $\overline{V}$ of the random sample were determined.

An essential point of this method is that the $\overline{SA}$ and $\overline{V}$, which are derived from ΣSA and ΣV, are not the same as the SA and V of a particle of mean diameter. This point has been stressed in a review by Dole and Hamlin (1962):

> At the risk of overemphasizing technical difficulties, it must be mentioned that chemical analysis of a particle mixture, even apart from problems of contamination and loss, gives little information about the structure of the surface layer. The snag lies in the heterogeneity of particle size. Even if all particles could be assumed to be similar in structure, and all the surface-active molecules could be assigned to the surface, one could not calculate the average thickness of the surface coat without knowledge of the size distribution. (continued on p.63)

TABLE 2

Reference table of measurements of spherical particles

Particle diameter		S.A.	V.
on plate in micrometer units	original diameter $\overset{o}{A}$	(πd^2) $10^7 \overset{o}{A}^2$	$(\frac{\pi d^3}{6})$ $10^9 \overset{o}{A}^3$
1	120	.0045	0.0009
2	240	.0181	0.0072
3	360	.0407	0.0245
4	480	.0724	0.0579
5	600	.113	0.113
6	720	.162	0.195
7	840	.221	0.310
8	960	.289	0.463
9	1080	.366	0.660
10	1200	.452	0.905
11	1320	.547	1.204
12	1440	.651	1.563
13	1560	.764	1.988
14	1680	.887	2.483
15	1800	1.018	3.054
16	1920	1.158	3.706
17	2040	1.308	4.445
18	2160	1.465	5.277
19	2280	1.633	6.206
20	2400	1.810	7.238
21	2520	1.995	8.379
22	2640	2.10	9.63
23	2760	2.38	11.01
24	2880	2.61	12.51
25	3000	2.83	14.14
26	3120	3.06	15.94
27	3240	3.30	17.85
28	3360	3.55	19.85
29	3480	3.81	22.05
30	3600	4.07	24.41
31	3720	4.35	26.90
32	3840	4.62	29.70
33	3960	4.92	32.61
34	4080	5.24	35.55

(continued p.63)

TABLE 2 (continued)

Particle diameter		S.A.	V.
on plate in micrometer units	original diameter $\overset{o}{A}$	(πd^2) $10^7 \overset{o}{A}^2$	$(\frac{\pi d^3}{6})$ $10^9 \overset{o}{A}^3$
35	4200	5.54	38.85
36	4320	5.86	42.35
37	4440	6.17	45.70
38	4560	6.52	49.65
39	4680	6.87	53.60
40	4800	7.24	57.95

(continuation from p.61)

It is not accurate to express the data in terms of an average particle, since the surface: volume relation of an average unit differs from the total surface : total volume relation for the mixture. To see this, consider a mixture of particles, 99 per cent with a diameter of 100 mμ, and 1 per cent of 500 mμ. The large particles although few in number, contribute 56 per cent of the mass, and 20 per cent of the surface. The arithmetic average of the diameters is 104 mμ; the diameter of a "weight average" particle is 127 mμ. Corresponding to these two different kinds of average particle, the surface : volume ratios are 28.8 and 23.6μ^{-1}, respectively. The actual ratio of total surface to total volume (the value given by analytical data) is only 16.6μ^{-1}. If all the particles in this hypothetical mixture were in fact covered by a monomolecular film the low value of the analytical ratio, interpreted in terms of an average particle, would lead to the false conclusion that not enough surface material was present to form a complete coat.

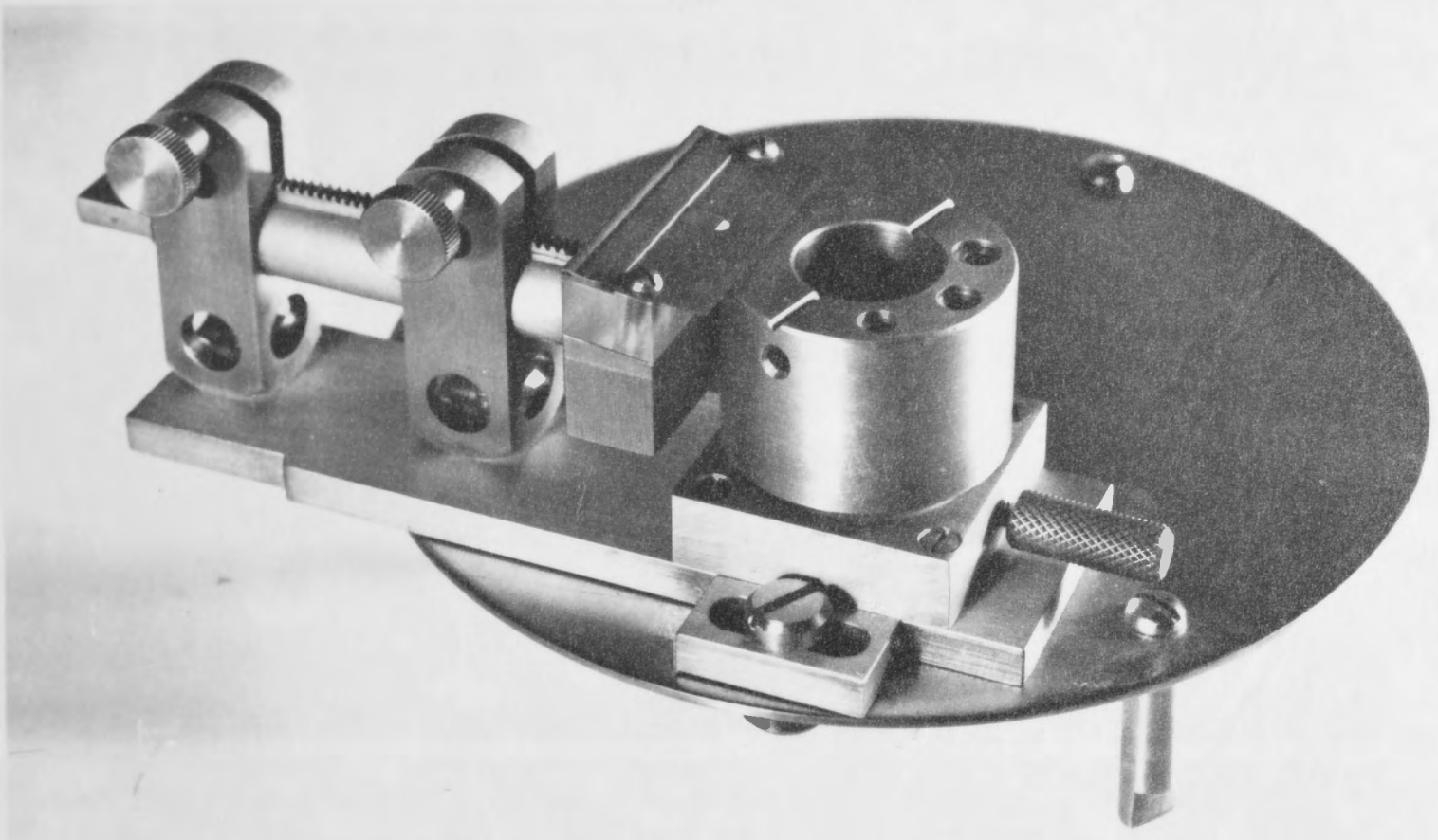
(iv) Electron microscopy of chylomicrons from rabbits fed butter

Electron microscopy of chylomicrons from butter fed animals has to date been unsuccessful (Jones et al, 1963; Bierman et al, 1966). The difficulty is in the fixation of saturated fat since the fatty acids of chylomicrons tend to reflect the fatty acid pattern of dietary triglycerides (Dole and Hamlin, 1962; Karmen et al, 1963) and the fixation of lipid by osmium probably depends on the degree of unsaturation (Schoefl, 1968).

Freeze etching of specimens with carbon replication and shadowing (Steere, 1957) is a method of preparation of specimens for electron microscopy without the necessity of chemical fixation. This method has further been developed by Moor et al (1961) and a commercial unit has been developed by Balzers Aktiengesellschaft in the Principality of Liechtenstein. The principles of this method might enable the preparation of chylomicrons for electron microscopy and a modified apparatus, designed by Dr Cliff, is shown and described in Fig. 6. The resultant electron micrographs are shown in Fig. 7. The results are as yet not always reproducible, so no size estimations have been attempted on chylomicrons from butter fed rabbits.

Figure 6

The apparatus used for freeze etching techniques.



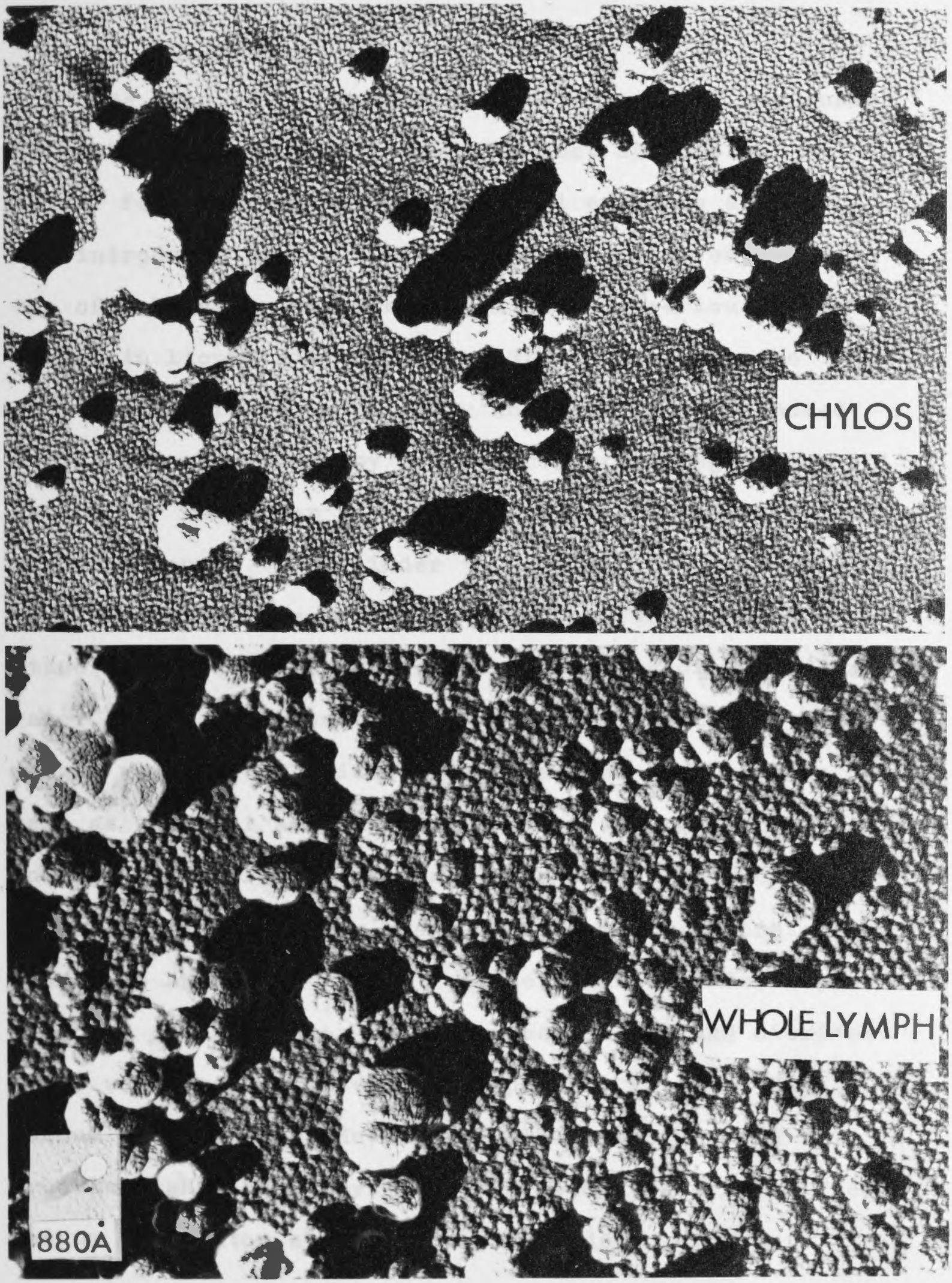
The brass cylinder has side holes for gripping by forceps to enable it to be plunged into liquid nitrogen. The central well holds liquid nitrogen and the peripheral holes receive the drops of chylomicron emulsion. The cylinder is then rotated through 90 degrees to bring the specimen to near the microtome blade and to adjust their relative height.

The whole apparatus is then placed in a vacuum evaporator before slicing, etching, carbon replication and shadowing of the specimen.

Figure 7

Chylomicrons (above) and whole lymph (below) from the thoracic duct lymph of a rabbit fed 30 per cent butter. The specimens have been prepared by freeze etching techniques

BUTTER DIET



a) Procedure

One drop of the specimen, consisting of a suspension of chylomicrons from the thoracic duct lymph of a butter fed rabbit or a soya bean oil emulsion as a control, was introduced over a small hole in the flat edge of the top of a brass cylinder block which had previously been cooled in liquid nitrogen. The drop containing the chylomicrons froze immediately and was firmly anchored to the brass cylinder by its lower portion pegged in the hole. The upper part of the pellet protruded above the flat top of the cylinder to a height of about 0.5 cm.

The cold brass cylinder was then attached to a stage which carried a microtome consisting of a sliding razor blade. Between the cylinder and the stage a plastic washer was present which acted as a heat insulator as well as a height adjuster by means of a screw thread on the bottom of the cylinder. The height of the cylinder had previously been adjusted so the microtome blade was approximately 0.3 cm above the cylinder top.

The cold cylinder with specimen pellet and microtome stage was placed in a vacuum evaporator (Dynavac High Vacuum Coating Unit, Model CE 12/14A, manufactured by Dynavac High Vacuum Pty. Ltd., Burwood, Victoria). The vacuum evaporator contained carbon rods to evaporate

carbon, a tungsten filament for evaporation of chromium, and a lever attached to the microtome to permit movement of the blade. Within the vacuum evaporator was a cold trap consisting of a brass vane of about 30 cm^2 area connected to a liquid nitrogen cold finger. The cold vane was located near the specimen pellet and was angled to permit the evaporated chromium to fall on the specimen surface at an angle of about 15 degrees. A small hole was situated in the vane's centre so as not to obstruct the passage of carbon from the carbon arc to the specimen.

After creating a vacuum of better than 10^{-5} mm Hg in the evaporator and cooling the cold trap with liquid nitrogen, the microtome was manoeuvred to crack off the top of the pellet of chylomicrons and to leave a clean horizontal surface of the specimen facing the cold trap. At this stage etching theoretically took place since the cold trap was at a lower temperature than the frozen specimen allowing the sublimation of ice from the surface of the specimen to the cold trap and so leaving the lipid particles protruding from the surface. The time of etching was varied from one minute to ten minutes.

The specimen was then lightly coated with carbon to form a replica of its etched surface and was then

shadowed at an angle of 15 degrees with chromium to give the replica a three dimensional effect.

The brass cylinder with the specimen pellet and surface replica was then removed from the vacuum evaporator and allowed to come to room temperature. The carbon replica was lifted from the surface of the now liquid drop with a fine specimen grid and washed in acetone and chloroform-methanol. The specimen was then ready for examination in the electron microscope.

b) Improvements yet to be incorporated

The most likely source of variation is in the etching process. The brass cylinder holding the specimen is at a variable temperature depending on the time since it has been in liquid nitrogen and the speed vacuum is established in the evaporator. To obtain reproducible etching the temperature of the specimen, the temperature of the heat trap, the distance of the heat trap from the specimen and finally the etching time need to be controlled. To control these variables adequately it is intended to install thermocouples within the brass cylinder and heat trap.

c) Summary

The freeze-etching technique differs from customary methods of fixation and cutting in that it is a purely

physical preparation of the specimen. The method consists mainly of four preparative steps. After freeze fixing the specimen is cut in the vacuum chamber, etched by low temperature sublimation and a replica of the surface produced by coating with carbon. The structure and chemical composition of the specimen theoretically remain unchanged.

7. Techniques used for analysis of individual lipids

This section deals with techniques which are well documented in the literature. Only a summary of the principles of the methods and certain modifications and difficulties encountered will therefore be mentioned.

(i) Extraction and purification of the lipid sample

(Folch et al, 1951 and 1957). This method consists of breaking down the lipoproteins and extracting the lipid from the specimen by the addition of 20 volumes of chloroform-methanol (2:1 by volume) to 1 volume of specimen. The extract is then washed by the addition of excess water to remove all the non-lipid contaminants.

The only difficulty found was the loss of phospholipid to the water phase. This was overcome when all glassware was acid washed, not washed in detergent.

(ii) Analysis of total cholesterol (C)

a) saponification (Abell et al, 1952)

An aliquot of lipid extract, or a small volume of serum (0.1 or 0.2 ml) was saponified in alcoholic potassium hydroxide to break down lipoproteins and to release cholesterol and cholesterol esters for hydrolysis to free cholesterol. The total cholesterol (now as free cholesterol) was taken up in 10 ml of petroleum ether.

b) Zak colour development (Zak et al, 1954; Herrmann, 1957)

The method depends on colour development by the interaction of free cholesterol with ferric chloride in the presence of acetic and sulphuric acid. The optical densities of specimens were compared in a Beckman spectrophotometer at a wave length of 560 mμ.

c) Standard used and accuracy of method

Highly purified ash free cholesterol known as "bacto-cholesterol" manufactured by the Difco Laboratories of Michigan U.S.A. was used. The standard resulted in a straight line curve of optical density when 50, 100 and 150 μg of cholesterol were prepared by firstly saponifying 500 μg of

cholesterol, taking this up in 10 ml of petroleum ether and then developing colour from 1, 2 and 3 ml of this solution.

Each batch of readings was also checked with a control of "Choles-trol" (Dade Reagents Inc., Miami, U.S.A.) stated to contain 218 mg cholesterol/100 ml. The method described gave a mean concentration of 230 $\pm$ 2.5 mg/100 ml from 40 separate readings.

d) <u>β- sitosterol</u>

The colour development of β- sitosterol (50,100 and 150 μg) (Calbiochem. Los Angeles, U.S.A.) gave the same curve of optical densities as the cholesterol standard, with maximal light absorption also at 560 mμ wave length.

(iii) <u>Triglyceride (TG) estimation</u> (Van Handel and Zilversmit, 1957)

This method depends on the colour developed from the interaction of chromotropic acid with formaldehyde derived from the glycerol of triglyceride.

The first step necessary therefore was to remove glycerol from sources other than triglyceride. This was achieved by first dissolving the lipid sample or aliquot in chloroform, no methanol being present, and

washing the sample with chloroform through a silicic acid column (1 g of 1:1 non-activated silicic acid : supercel) to remove phospholipid. The resultant lipid fraction (free of phospholipid, as was checked on a number of occasions) was then saponified to release glycerol from the triglyceride. The glycerol was then converted to formaldehyde by the addition of sodium periodate. A colour was then developed by the addition of chromotropic acid and optical densities of the samples were then compared with a Beckman spectrophotometer.

Standards were prepared from corn oil of known mass dissolved in chloroform and treated in the same way as the sample. At optical density of 570 mμ standards of 25, 50, 75 and 100 μg of triglyceride (corn oil) gave a straight line curve.

(iv) Phospholipid (PL) estimation

(Bartlett, 1959 and Morrison, 1964)

This estimation depends on measuring the inorganic phosphorus of the sample and converting this by a constant factor (x25) to estimate the mass of lecithin from which it was derived. The factor is obtained by dividing the molecular weight of lecithin (approximately 775) by the atomic weight of phosphorus (31). This pre-supposes constant fatty acids in the lecithin

molecule, and that all the phospholipid of the sample is lecithin. The factor of x 25 is slightly inaccurate when measuring other phospholipids. For example cephalin has a molecular weight of about 750, depending on the fatty acids present, and sphingomyelin of about 720 in lymph when mainly 16 chained fatty acids are present but in serum about 800 since mostly 24 chained fatty acids are present (Zilversmit, 1968a). In lymph, however, more than 70 per cent of phospholipid is lecithin with cephalin and a little sphingomyelin making up the remainder (Whyte et al, 1963).

In summary the method consisted of first heating the sample for 1.5 hr at 200°C with concentrated sulphuric acid to convert the organic phosphorus of the phospholipid to phosphoric acid. The phosphoric acid was then estimated by adding ammonium molybdate and Fiske Subba Row colour reagent. The samples were then compared for optical density in a spectrophotometer at 820 mμ wave length.

The standard stock solution was made up of 4.391 g of KH_2PO_4/litre of water and was diluted by $\frac{1}{500}$ to give a dilute standard containing 2 μg P/ml. Readings of 1 μg, 2 μg and 3 μg of phosphorus standard gave a straight line curve of optical densities. Pure lecithin

was not available so the method was checked against an organic phosphate by using di(ethyl hexyl) phosphoric acid (M.W. 322) as a control.

8. Methods used for the study of clearing factor lipase including isotopic labelling techniques

(i) Preparation of labelled chylomicrons from thoracic duct lymph

Rabbits of 2-3 kg were fed diets of 30 per cent corn oil and 0.8 per cent cholesterol in powdered rabbit food for about a week before operation. On the morning of operation 500 microcuries of ^{3}H-palmitic acid (palmitic acid-9,10-TCn) supplied by Radiochemical Centre, Amersham, England) was given by capsule to two rabbits (R161 and R167) two hours before operation. To another two rabbits (R169 and 171) 50 microcuries of ^{14}C-cholesterol (cholesterol-4-^{14}C) was given by the same method and to a fifth rabbit (R172) both 500 microcuries ^{3}H-palmitic acid and 50 microcuries ^{14}C-cholesterol were given. The rabbits were then lightly anaesthetised and thoracic duct lymph obtained by the method of Zilversmit *et al* (1967). The lymph was allowed to clot, the clot removed with a wooden applicator stick and the chylomicron fraction ($S_{f>400}$)

separated by ultracentrifugation. The chylomicron fraction was then reconstituted to its original volume by careful resuspension in 1.1 per cent saline.

(ii) Preparation of normal serum and clearing factor lipase serum

Rabbits were bled about an hour before need of the serum. A sample of 10 ml of blood was first obtained from the central ear artery of the rabbit and the serum for this taken as the control. Then 0.5 ml heparin (500 I.U.) was injected into the rabbits' marginal ear vein and after twenty minutes a further 10 ml of arterial blood was taken. This post-heparin serum contained the clearing factor lipase and will be known as CF serum. To both these serum samples 100 I.U. heparin were added to prevent any further clotting as the CF serum otherwise had a tendency to further clotting during incubation with chylomicrons.

(iii) In vitro incubation of chylomicrons and CF serum

For each experiment 1 ml of reconstituted lymph chylomicrons was added to 4 ml of serum, both control serum and CF serum, and 0.3 g bovine serum albumin added to each as an acceptor for free fatty acid.

Optical densities at wave length 560 Å were immediately noted and after one hour incubation at 37°C the OD_{560} was again checked to confirm clearing with the CF mixture but not with the control.

(iv) Post incubation ultracentrifugation of chylomicron-serum incubation mixture

The serum-chylomicra mixtures (CF and control) after the one hour incubation were then subjected to ultracentrifugation at 4°C. During this procedure further lipase activity probably occurred in the CF serum-chylomicron mixture. The mixtures were divided into three fractions, namely chylomicrons or $S_{f>400}$, VLDL or $S_{f12-400}$ and D>1.019 containing low density and high density lipoproteins, albumin-FFA complexes if present. As well as the three main fractions, from the second spin in the preparation of the chylomicron fraction two further fractions were sliced so as to retain all the radioactivity. This consisted of the saline layer of 1.006 g/ml through which the chylomicrons were washed and which would contain mainly VLDL (designated subnatant 1) and the bottom dense layer in the tube consisting mainly of D>1.019 lipoproteins, albumin and albumin-free fatty acid complexes and also some VLDL (designated subnatant 2).

(v) Optical densities of the fractions obtained by ultracentrifugation

All fractions were made up to 5 ml in 1.1 per cent saline for comparison of OD_{560} from the control serum mixture and CF serum mixture in a Beckman spectrophotometer.

(vi) Relative radioactivity in the fractions

The radioactivity in each fraction was estimated in a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3003) after extraction with chloroform:methanol (2:1). The lower phase containing the lipid and radioactivity was pipetted into scintillation vials, dried under nitrogen, and a scintillation fluid consisting of 3 g of PPO (2,5-diphenyloxagole) to 1 litre of toluene was added. Background counts of normal lymph or serum extracts were subtracted. The radioactivity of each fraction was expressed as a percentage of the total in each sample. The difference in radioactivity between the control and CF serum of each fraction was also noted.

(vii) Triglyceride and cholesterol estimation of the chylomicrons

Thoracic duct chylomicrons from four other rabbits were incubated for varying times with CF serum and control serum in the same manner as described above and the chylomicron fractions obtained in a like manner. The

triglyceride (TG) (expressed as mg/100 ml) and total cholesterol (C) (mg/100 ml) were estimated and the $\frac{TG}{C}$ ratios compared.

(viii) Electron microscopy

The lipoproteins of the various fractions from the control serum mixture and CF serum mixture were fixed with 1 per cent osmium tetroxide and shadowed with chromium.

9. Methods used for other experiments involving isotopically labelled lipoproteins

(i) The distribution of radioactive cholesterol in thoracic duct lymph

Rabbits were fed diets of 0.8 per cent cholesterol with or without the addition of 30 per cent triglyceride. Capsules containing ^{14}C-cholesterol were fed 6 hr before cannulation of the thoracic duct, and the lymph was then ultracentrifuged into chylomicrons, VLDL and D>1.019. The radioactivity of the reconstituted fractions was measured in a scintillation counter.

(ii) The disappearance of labelled lipoproteins from the blood stream

Thoracic duct lymph chylomicrons or VLDL labelled in the triglyceride or cholesterol moities were

injected into the marginal ear veins of rabbits. The radioactivity of serial samples of serum was then measured over a period of five hours. Rabbits of 2 kg weight on continuing diets of 0.8 per cent cholesterol with or without 30 per cent triglyceride were fed one dose of ^{14}C-cholesterol and the radioactivity of serial samples of their serum measured for three weeks.

10. <u>The preparation of arterial specimens</u>

Autopsy material was obtained from rabbits fed 400 mg of cholesterol daily, with or without added fat, at twelve weeks after the start of their diets.

The aortae were opened with fine scissors along their ventral aspect and pinned to a hard plastic holder before fixing for 2 days in formol saline. They were then rinsed in water for 10 minutes, placed in 70 per cent alcohol for a further 5 minutes and then stained in Fettrot for about 10 seconds. After washing thoroughly in 70 per cent alcohol, and again in water they were stored in formol saline. The Fettrot stain was a filtered saturated solution in acetone to which an equal volume of 70 per cent alcohol had been added.

Specimens of heart and liver for light microscopy were fixed in formol saline for more than a week and were then embedded in gelatin (Carleton and Drury, 1957) for frozen sectioning. The sections were then stained for fat with Fettrot (Pearse, 1961) and for cholesterol by the Schultz histochemical adaptation of the Burchard-Liebermann reaction (Reiner, 1953; Weber et al, 1956; Cook, 1958 and Pearse, 1961).

RESULTS

A. SIZE AND COMPOSITION OF LIPOPROTEINS IN CHYLE

1. The diameters of the particulate lipid in the lipoprotein fractions of thoracic duct lymph

The diameter distribution of lipoproteins in chyle were determined in preliminary experiments in which triglyceride or cholesterol were added to the diet to increase the lipid content of thoracic duct lymph.

(i) Corn oil diet

Fig. 8 is a composite electron micrograph of lipid particles contained in lipoprotein fractions separated by the ultracentrifuge from the thoracic duct lymph of a rabbit fed 10 per cent corn oil. The percentage distributions of the diameters of a random 200 particles from each fraction are shown in Fig. 9.

The chylomicrons are the largest particles, ranging in this case from about 720 to 2,400 Å in diameter and as seen from their shadows are spherical in shape. The VLDL are smaller, ranging from about 360 to 840 Å in diameter while the lipoproteins in the D>1.019 fraction are still smaller with a maximum diameter of about 600 Å. The minimum diameter of D>1.019 lipoproteins is

Figure 8

Electron micrographs of particulate lipid at the same magnification from the chylomicron ($S_{f>400}$) VLDL ($S_{f12-400}$) and D>1.019 (high density and low density lipoproteins of S_f0-12) fractions from thoracic duct lymph of a rabbit fed 10 per cent corn oil. The polystyrene markers are 880 Å in diameter

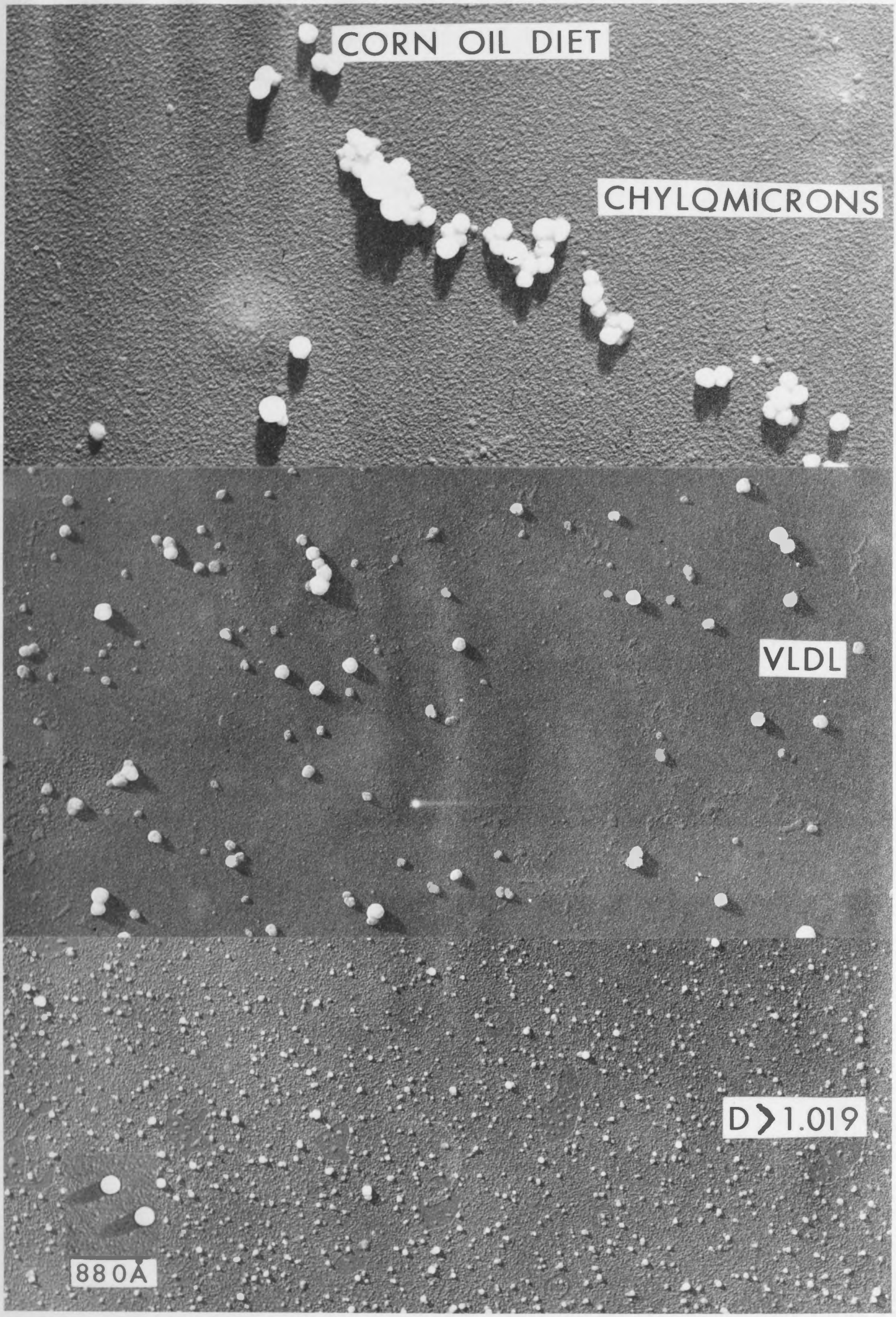
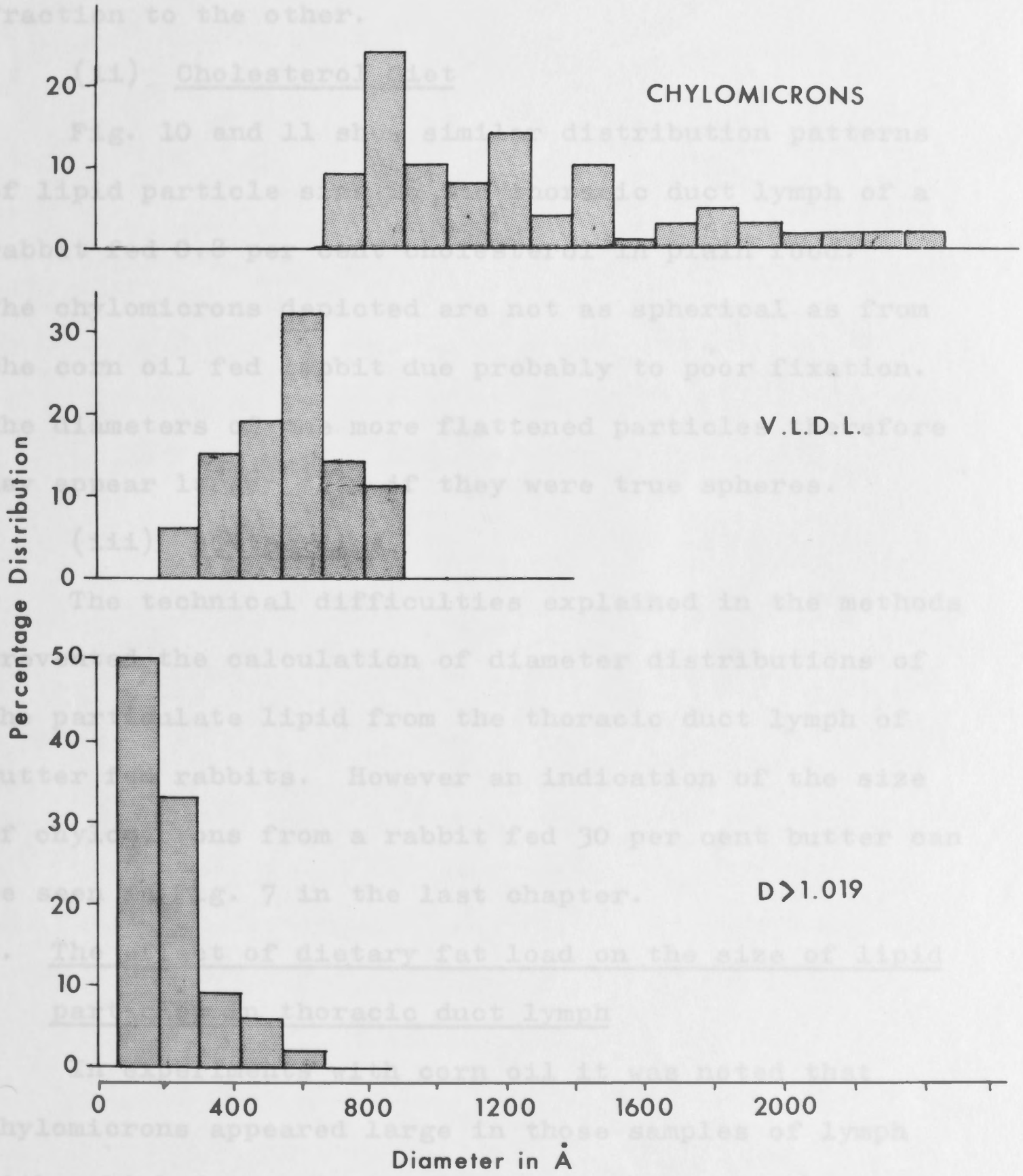

CORN OIL DIET
CHYLOMICRONS
VLDL
D > 1.019
880Å

Figure 9

The percentage distribution of diameters of particulate lipid from the chylomicron, VLDL and D>1.019 fractions of thoracic duct lymph from a rabbit fed 10 per cent corn oil

LIPROPROTEINS IN THORACIC DUCT LYMPH

CORN OIL DIET



hard to judge since the carbon-chromium membrane is rather granular but probably is about 120 Å. There is evidence of some overlap of particle diameters from one fraction to the other.

(ii) Cholesterol diet

Fig. 10 and 11 show similar distribution patterns of lipid particle size in the thoracic duct lymph of a rabbit fed 0.8 per cent cholesterol in plain food. The chylomicrons depicted are not as spherical as from the corn oil fed rabbit due probably to poor fixation. The diameters of the more flattened particles therefore may appear larger than if they were true spheres.

(iii) Butter diet

The technical difficulties explained in the methods prevented the calculation of diameter distributions of the particulate lipid from the thoracic duct lymph of butter fed rabbits. However an indication of the size of chylomicrons from a rabbit fed 30 per cent butter can be seen in Fig. 7 in the last chapter.

2. The effect of dietary fat load on the size of lipid particles in thoracic duct lymph

In experiments with corn oil it was noted that chylomicrons appeared large in those samples of lymph with a high fat content. This observation led to a more

Figure 10

Electron micrographs of particulate lipid at the same magnification from the chylomicron, VLDL and D>1.019 fractions from thoracic duct lymph of a rabbit fed 0.8 per cent cholesterol in plain food. The polystyrene markers are 880 Å in diameter

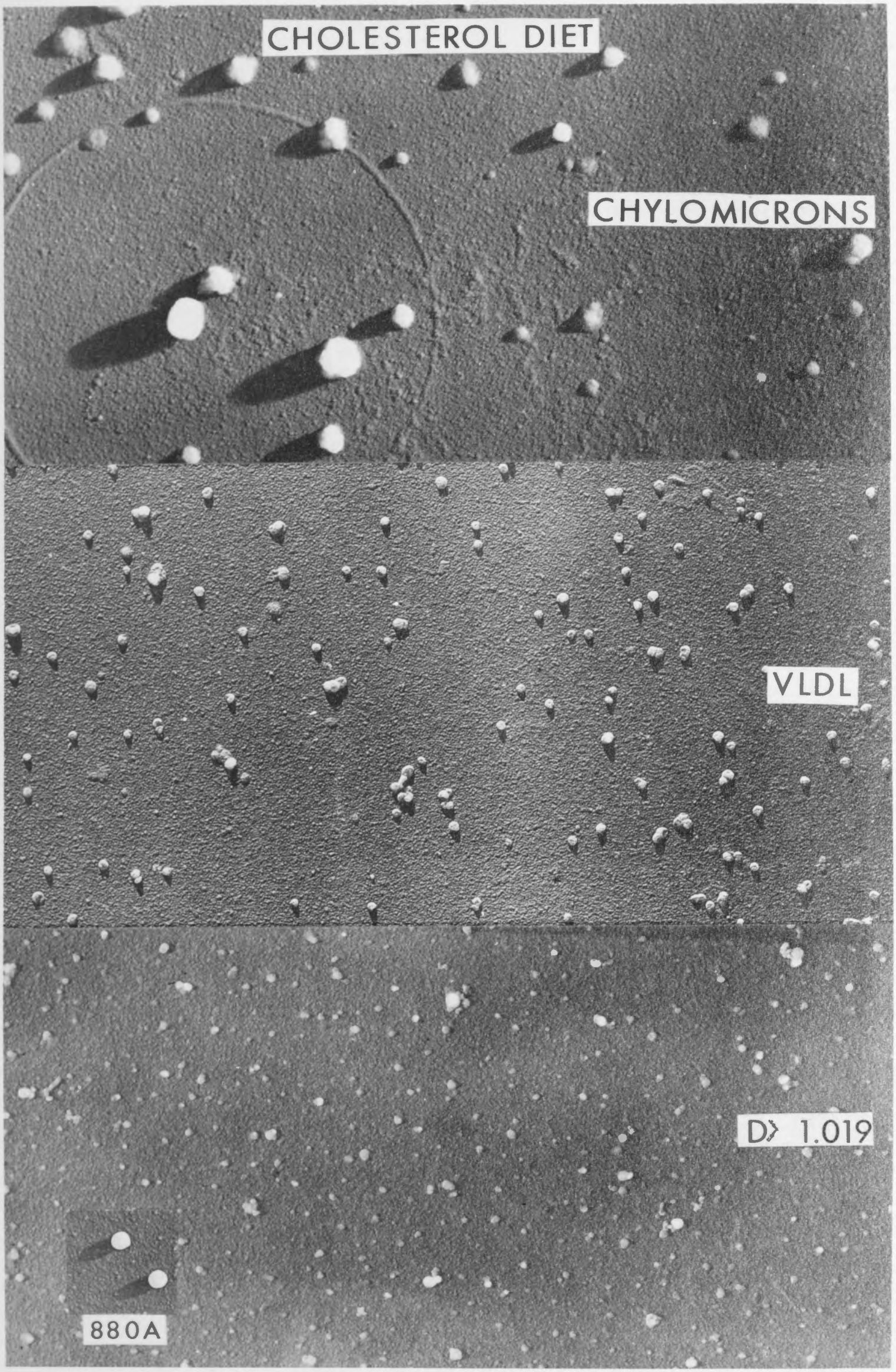
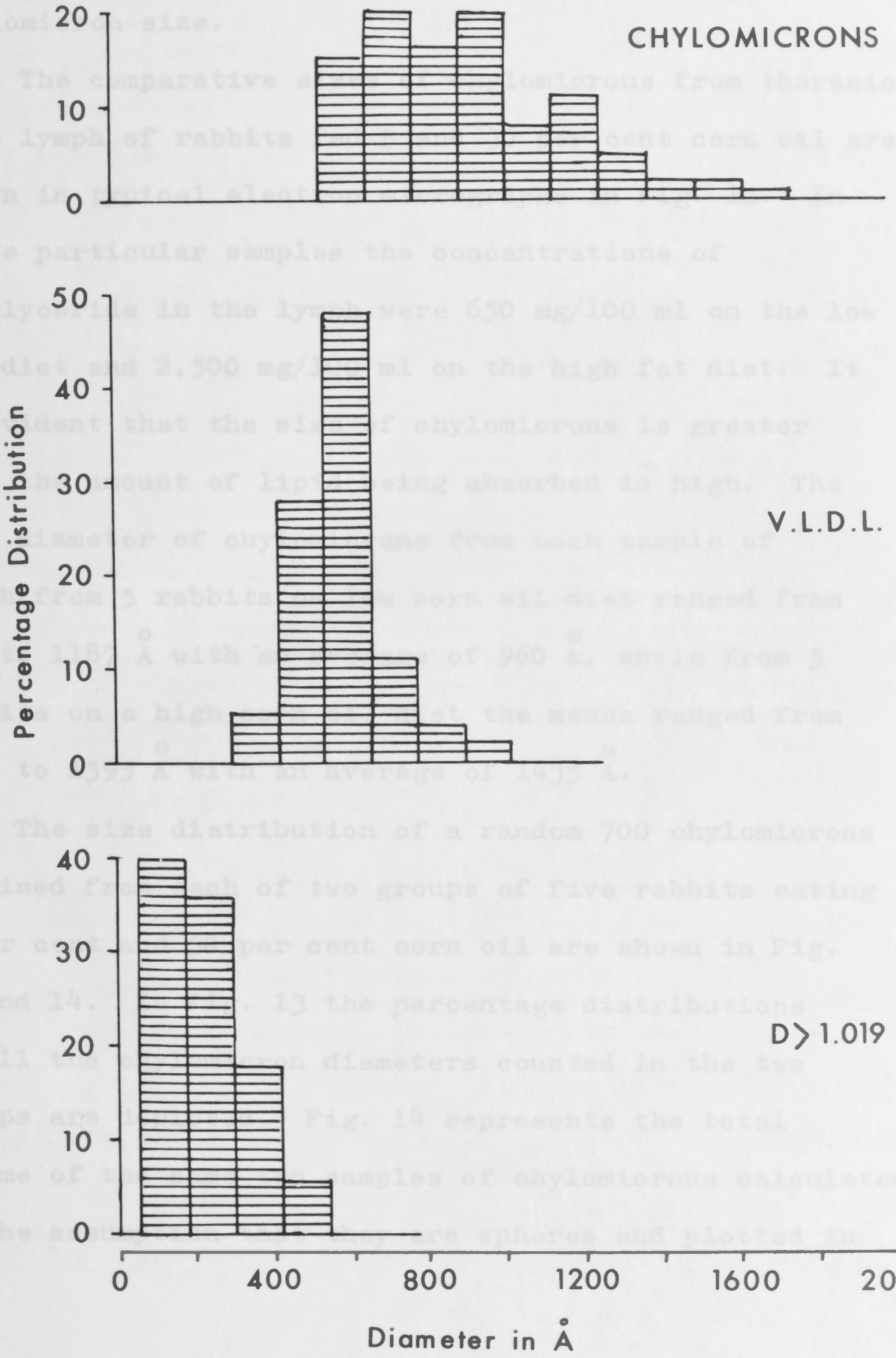

CHOLESTEROL DIET
CHYLOMICRONS
VLDL
D› 1.019
880A

Figure 11

The percentage distribution of diameters of particulate lipid from the chylomicron, VLDL and D>1.019 fractions from thoracic duct lymph of a rabbit fed 0.8 per cent cholesterol in plain food

LIPOPROTEINS IN THORACIC DUCT LYMPH

CHOLESTEROL DIET



systematic study of the effect of dietary fat load on chylomicron size.

The comparative sizes of chylomicrons from thoracic duct lymph of rabbits fed 5 and 30 per cent corn oil are shown in typical electron micrographs in Fig. 12. In these particular samples the concentrations of triglyceride in the lymph were 650 mg/100 ml on the low fat diet and 2,500 mg/100 ml on the high fat diet. It is evident that the size of chylomicrons is greater when the amount of lipid being absorbed is high. The mean diameter of chylomicrons from each sample of lymph from 5 rabbits on low corn oil diet ranged from 832 to 1187 Å with an average of 960 Å, while from 5 rabbits on a high corn oil diet the means ranged from 1235 to 1595 Å with an average of 1435 Å.

The size distribution of a random 700 chylomicrons obtained from each of two groups of five rabbits eating 5 per cent and 30 per cent corn oil are shown in Fig. 13 and 14. In Fig. 13 the percentage distributions of all the chylomicron diameters counted in the two groups are depicted. Fig. 14 represents the total volume of the same two samples of chylomicrons calculated on the assumption that they are spheres and plotted in

Figure 12

Electron micrographs of chylomicrons ($S_{f>400}$) in thoracic duct lymph from rabbits eating a diet containing (A) 5 per cent corn oil and (B) 30 per cent corn oil. The polystyrene markers are 880 Å in diameter

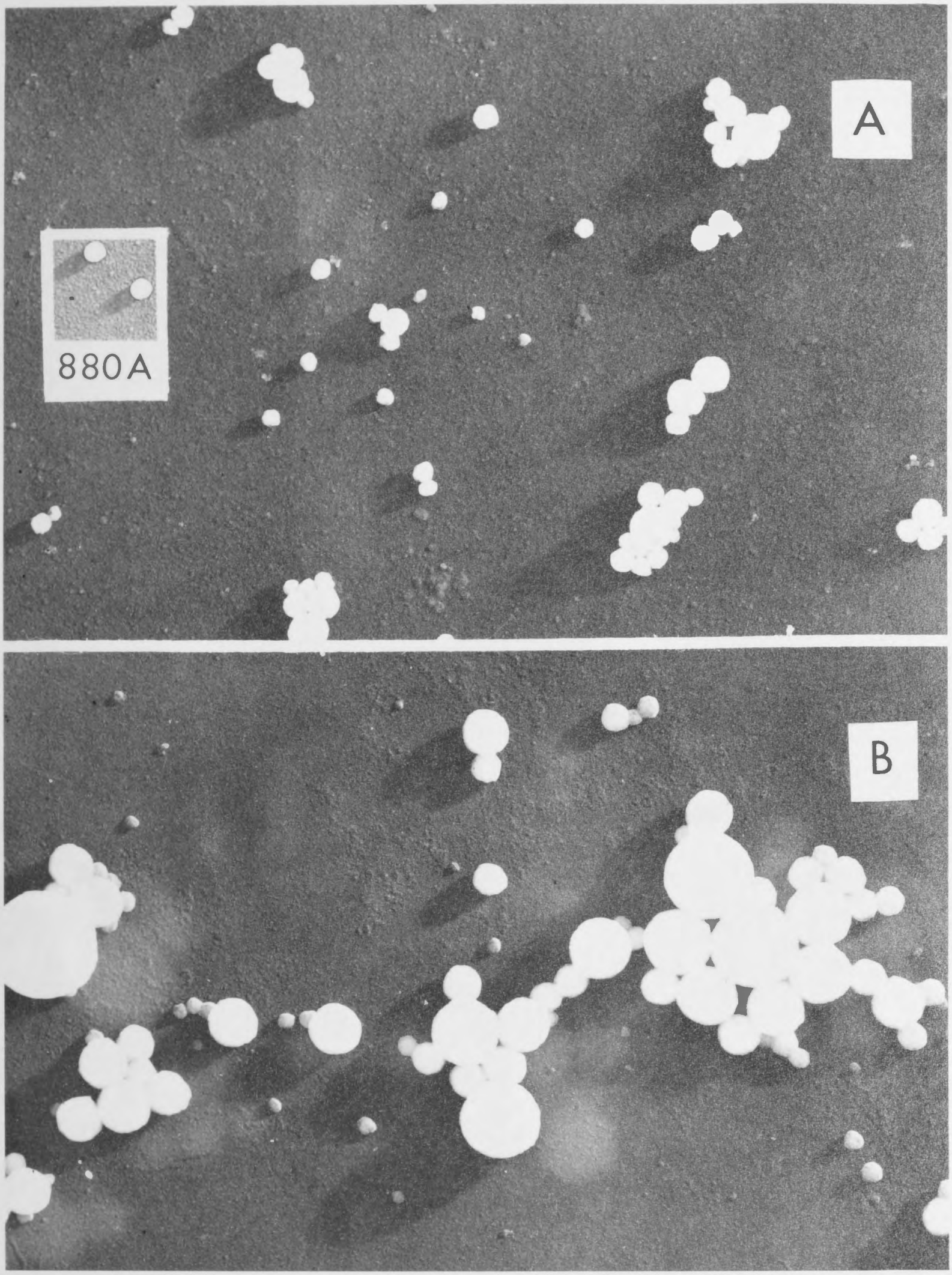

A
880A
B

Figure 13

The percentage distribution of chylomicrons according to their diameter, in thoracic duct lymph of rabbits fed a diet containing 30 per cent corn oil (upper graph) and 5 per cent corn oil (lower graph).

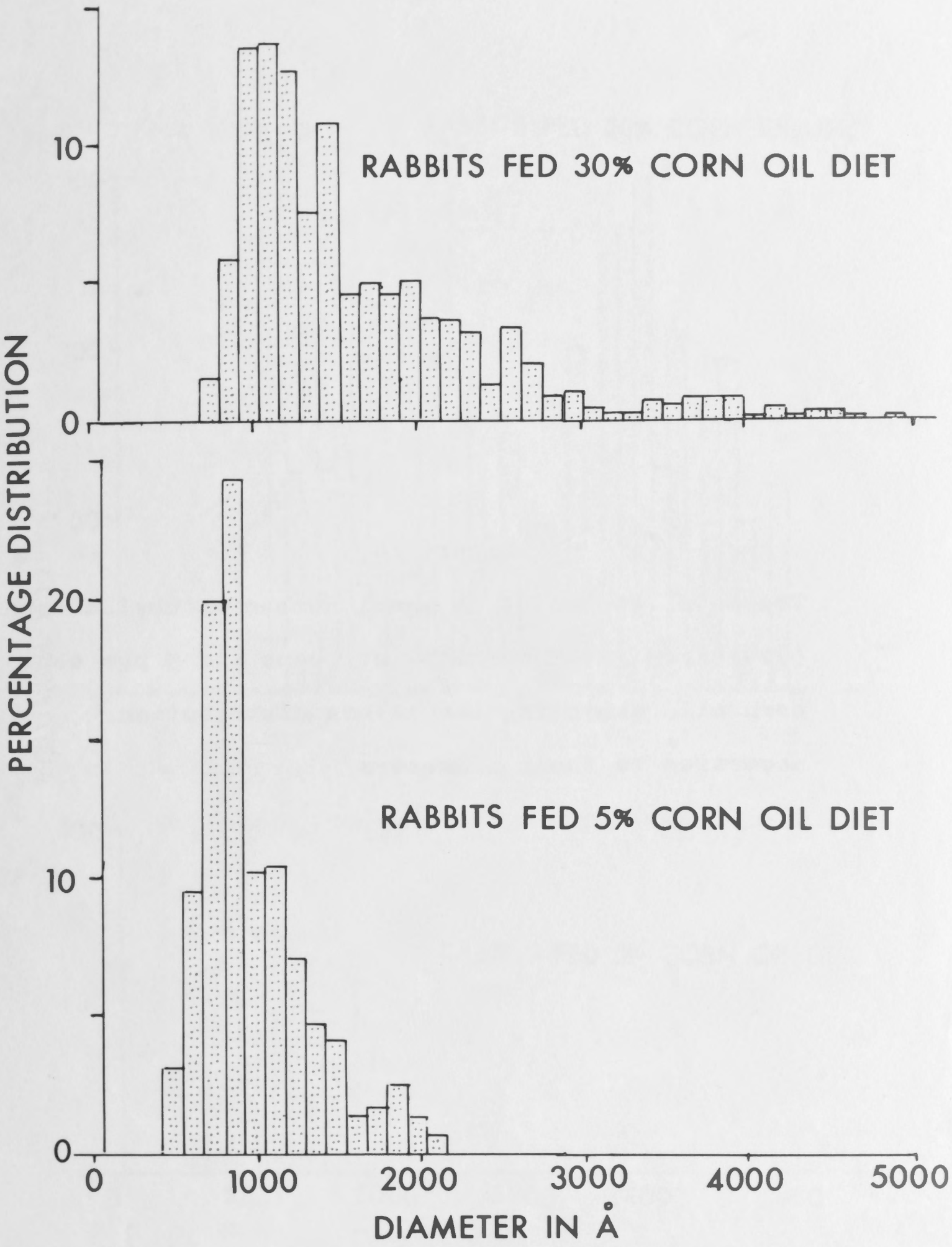
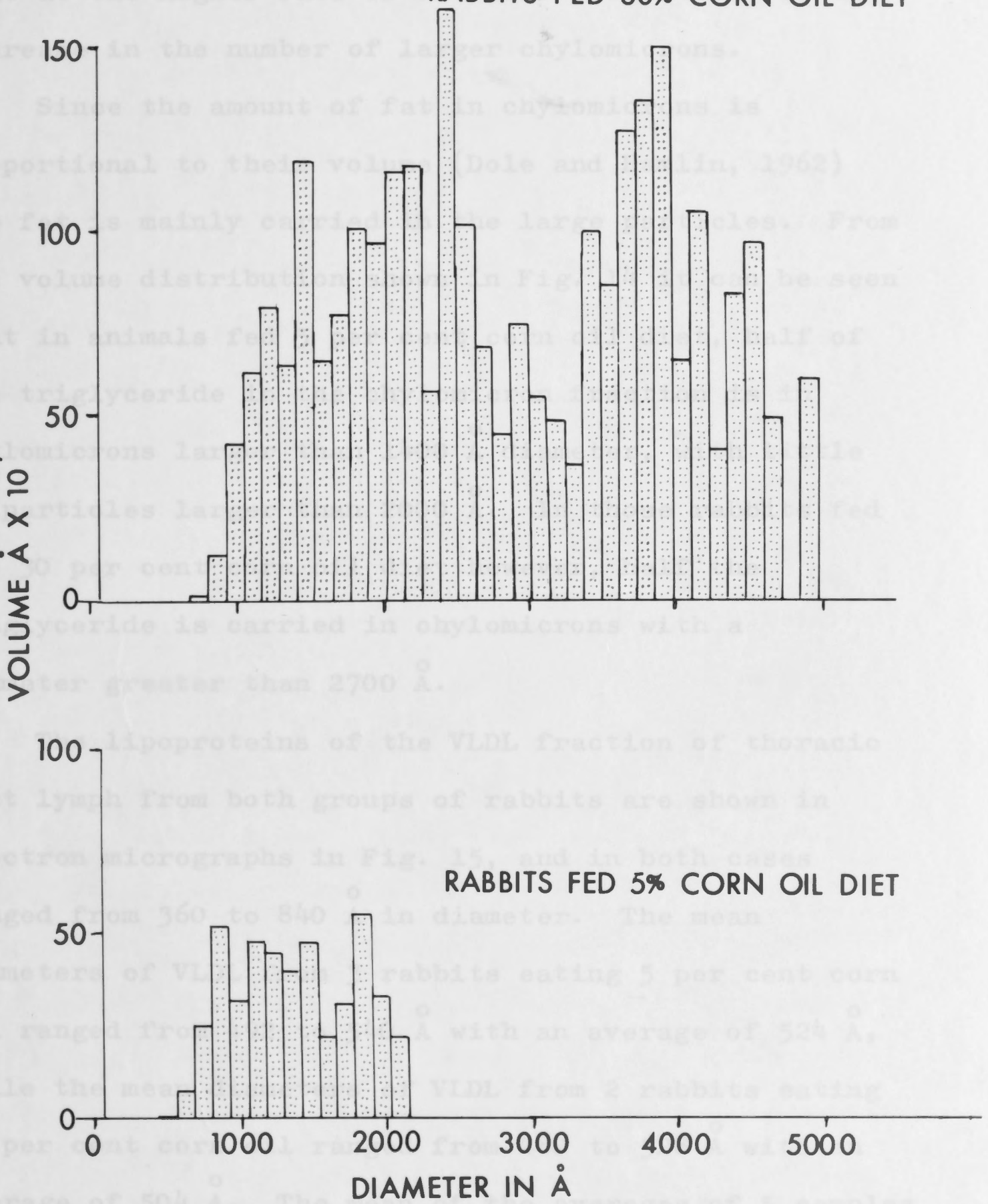

RABBITS FED 30% CORN OIL DIET
10
0
RABBITS FED 5% CORN OIL DIET
20
10
0
PERCENTAGE DISTRIBUTION
0
1000
2000
3000
4000
5000
DIAMETER IN Å

Figure 14

The total volume of an equal number of chylomicrons (700) from rabbits fed 30 per cent and 5 per cent corn oil, depicting the volume distribution according to their diameters


RABBITS FED 30% CORN OIL DIET
150
100
50
0
VOLUME Å X 10⁻⁹
RABBITS FED 5% CORN OIL DIET
100
50
0
0
1000
2000
3000
4000
5000
DIAMETER IN Å

relationship to their diameters. These figures show that at the higher rate of fat absorption there is an increase in the number of larger chylomicrons.

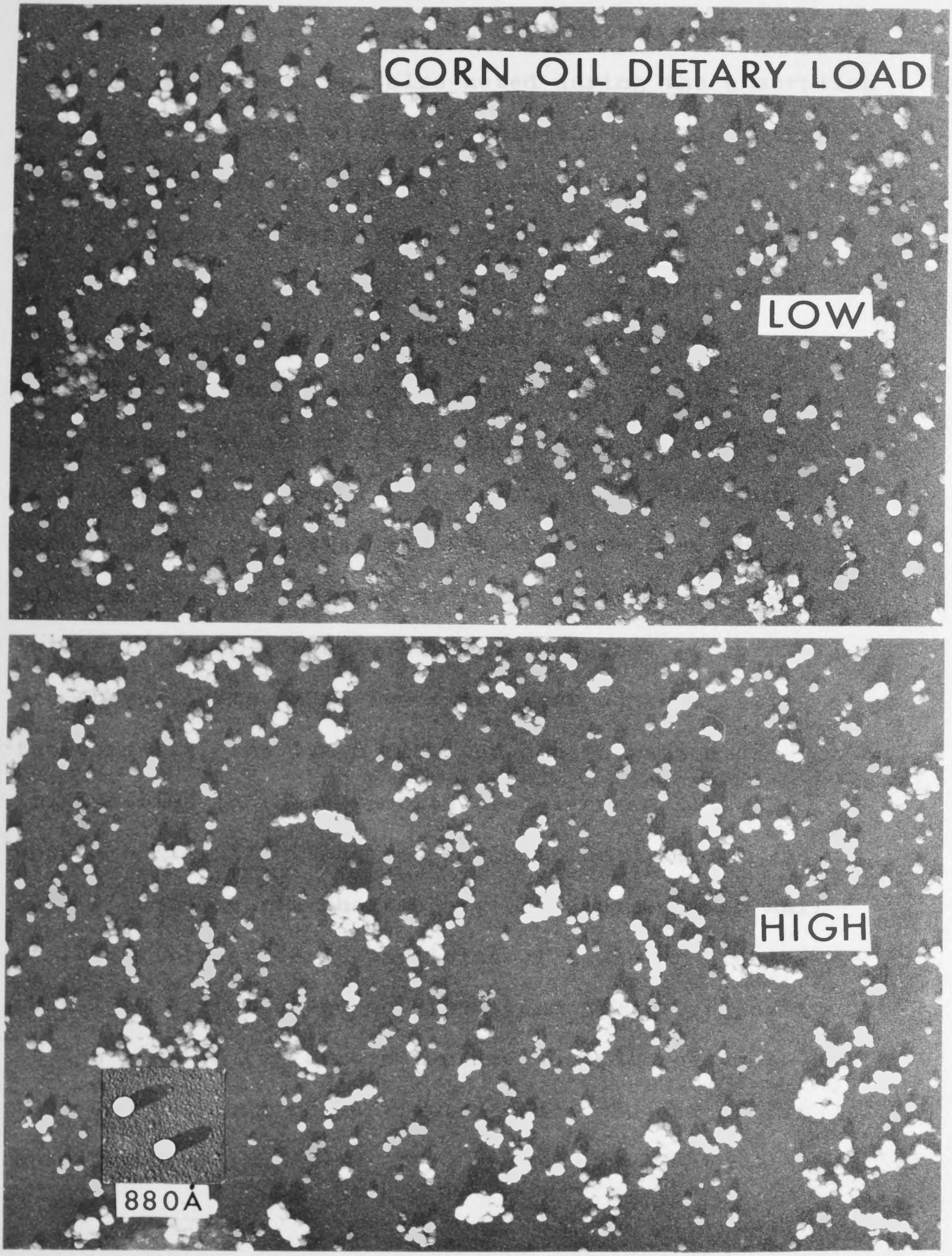
Since the amount of fat in chylomicrons is proportional to their volume (Dole and Hamlin, 1962) the fat is mainly carried in the large particles. From the volume distribution shown in Fig. 14 it can be seen that in animals fed 5 per cent corn oil diet, half of the triglyceride in the chylomicron fraction is in chylomicrons larger than 1400 $\mathring{A}$ diameter, with little in particles larger than 2000 $\mathring{A}$. In those rabbits fed the 30 per cent corn oil diet however, half the triglyceride is carried in chylomicrons with a diameter greater than 2700 $\mathring{A}$.

The lipoproteins of the VLDL fraction of thoracic duct lymph from both groups of rabbits are shown in electron micrographs in Fig. 15, and in both cases ranged from 360 to 840 $\mathring{A}$ in diameter. The mean diameters of VLDL from 3 rabbits eating 5 per cent corn oil ranged from 492 to 540 $\mathring{A}$ with an average of 524 $\mathring{A}$, while the mean diameters of VLDL from 2 rabbits eating 30 per cent corn oil ranged from 480 to 528 $\mathring{A}$ with an average of 504 $\mathring{A}$. The mean of the averages of 5 samples of VLDL was 516 ± 36 $\mathring{A}$.

Figure 15

Electron micrographs of VLDL in thoracic duct lymph from rabbits eating a diet containing 5 per cent corn oil and 30 per cent corn oil. The polystyrene markers are 880 Å in diameter

VLDL SIZE



In the rat an increase in chylomicron size from the thoracic duct lymph was also noted during the absorption of corn oil. A rat with a chronic thoracic duct fistula was fed a single intragastric dose of 0.5 ml corn oil and serial samples of lymph taken before, during and after fat absorption. As shown in Fig. 16 the lymph is at first comparatively clear but later during fat absorption becomes milky and opaque and eventually clears again as absorption is completed.

Composite electron micrographs of chylomicrons from some of the serial samples of lymph from the cisterna chyli of the rat obtained at various time intervals after a single intragastric dose of corn oil are shown in Fig. 17. The time after administration of the corn oil, the lymph flow and concentration of triglyceride in these samples are given in Table 3, opposite Fig. 17. The mean chylomicron diameter was 1094 $\overset{o}{A}$ in the early phase of fat absorption with half of the triglyceride in particles of diameter greater than 1320 $\overset{o}{A}$, increased to 2105 $\overset{o}{A}$ and 3600 $\overset{o}{A}$ respectively at the peak of fat absorption and then decreased to 1004 $\overset{o}{A}$ and 1200 $\overset{o}{A}$ respectively toward the end of fat absorption.

Figure 16

Serial samples of chyle from a rat fed 0.5 ml of corn oil by intra-gastric tube showing variations in optical density

Tube	Time after I.G. corn oil
1	0 - ½ hr after feeding corn oil
2	2¼-4¼ hr " " " "
3	4¼-6¼ hr " " " "
4	8¼-9¼ hr " " " "
5	9¼-10¼ hr " " " "

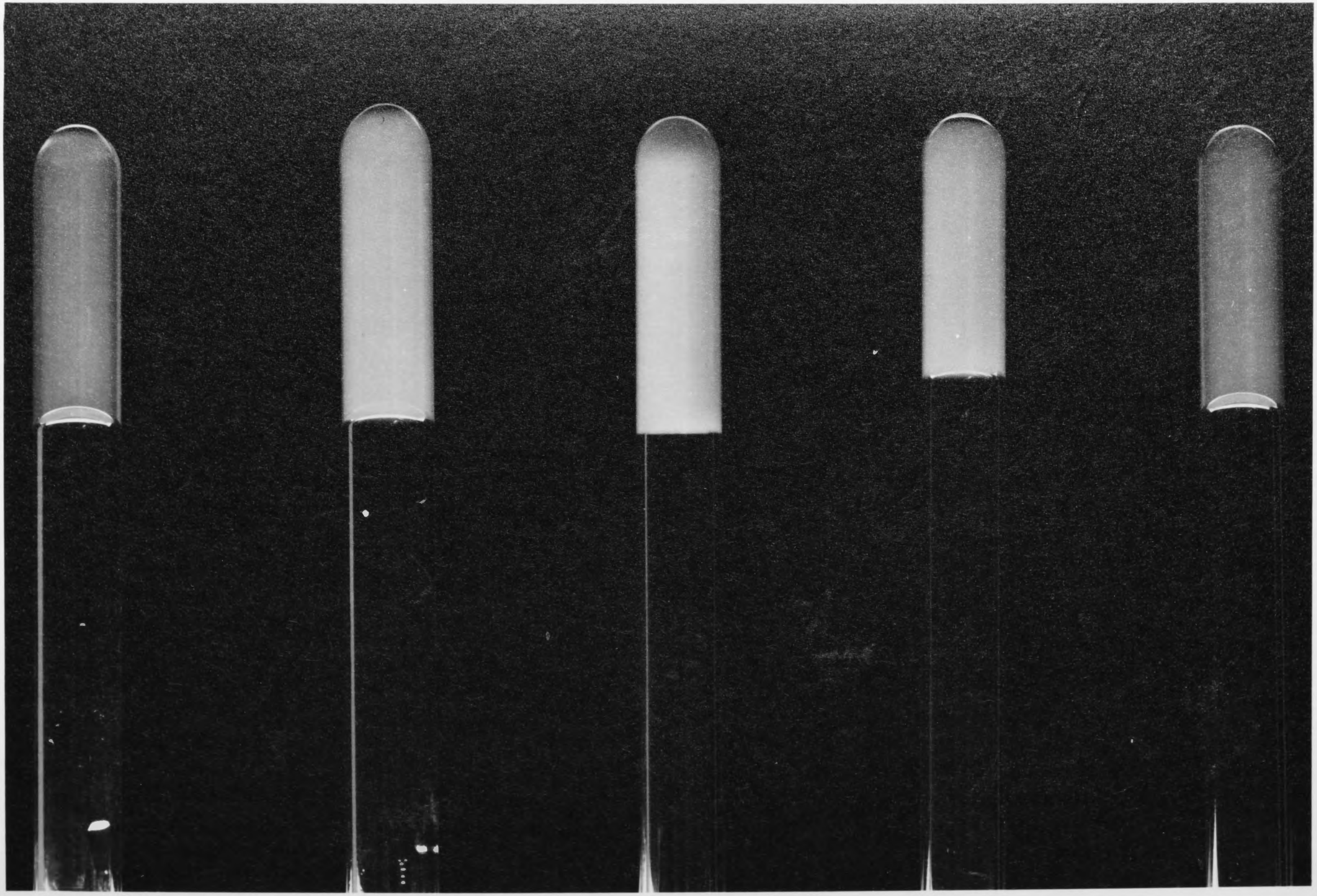


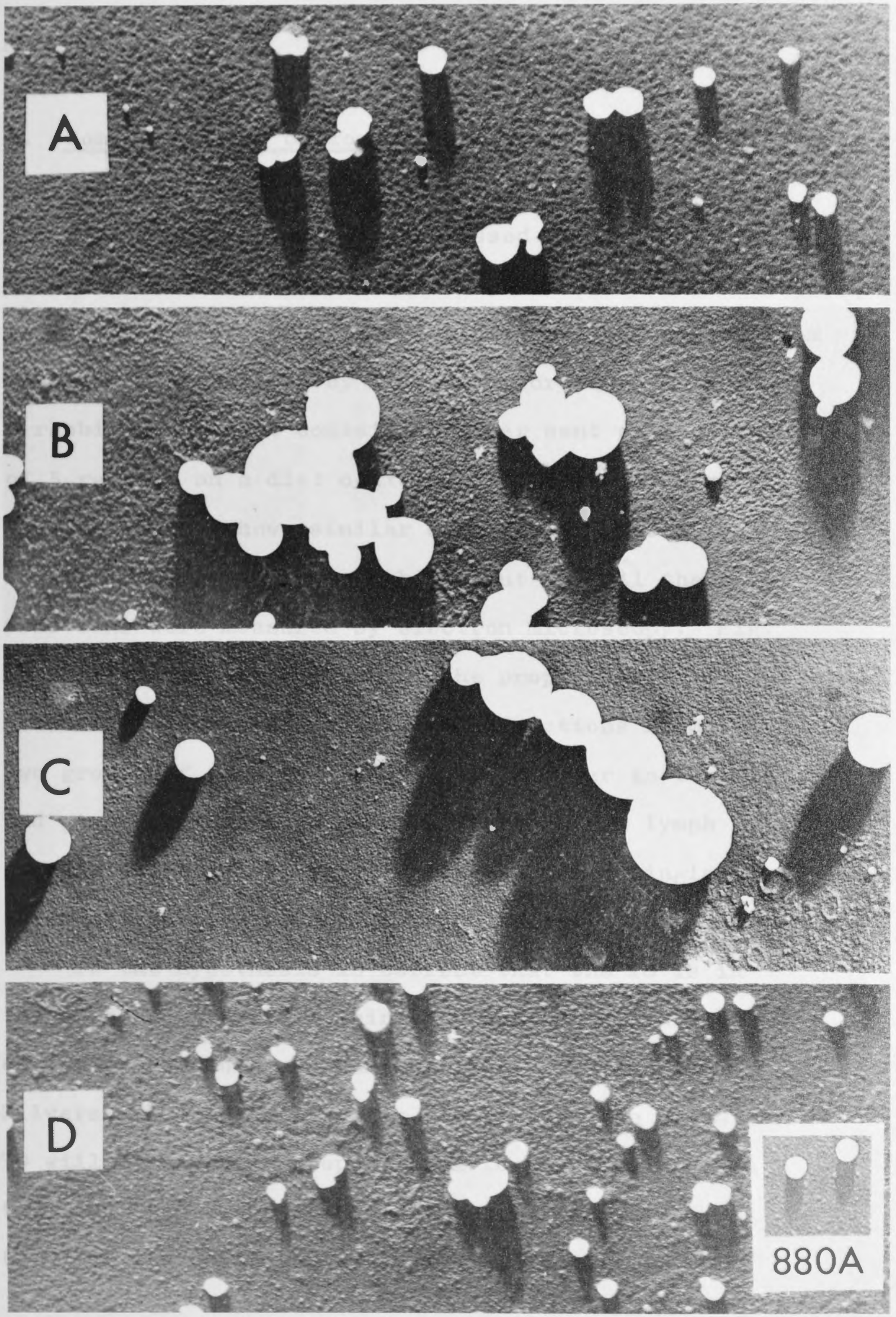
Figure 17
(opposite)

Electron micrographs of chylomicrons ($S_{f>400}$) in lymph from the cisterna chyli of a rat at various time intervals after giving a single dose (0.5 ml) of corn oil by stomach tube

TABLE 3
(below)

The flow and TG concentration of lymph obtained from the same rat. Also shown are the mean diameter and diameter of chylomicrons at the median volume. These samples correspond to those depicted in Fig. 17

Sample	Time after corn oil hr	ml/hr	mg/100 ml	diam. Å	Diam. of chylomicrons at median volume Å
A	0 - $\frac{1}{2}$	0.5	200	1094	1320
B	$2\frac{1}{4}$-$4\frac{1}{4}$	0.5	3400	1944	2760
C	$4\frac{1}{4}$-$6\frac{1}{4}$	0.7	8540	2105	3600
D	$9\frac{1}{4}$-$10\frac{1}{4}$	1.0	139	1004	1200


A
B
C
D
880A

3. Composition of chylomicrons in relation to their size

In Table 4 are shown the amounts of TG and PL in the chylomicron fractions, expressed in mg/100 ml lymph, together with the mean volume and surface area of a random sample of 100 or 200 chylomicrons calculated from measurements obtained by electron microscopy in each of 5 rabbits on a diet containing 5 per cent corn oil and of 5 rabbits on a diet containing 30 per cent corn oil. The Table also shows similar data for the VLDL ($S_{f12-400}$) fractions in lymph from rabbits, but not all these fractions were measured by electron microscopy. Fig. 18 diagramatically represents the proportion of TG found in the chylomicron and VLDL fractions from the two groups of rabbits. Table 5 is similar to Table 4 and gives measurements of chylomicrons from lymph samples collected at various times after a single intragastric dose of corn oil in the rat.

If the hypothesis is correct that the TG is in the core and the PL is thinly spread on the surface of chylomicrons (Dole and Hamlin, 1962; Yokoyama and Zilversmit, 1965; Zilversmit, 1965) then the amount of TG will be directly proportional to the total volume (ΣV) and the amount of PL to the total surface area (ΣSA) of the chylomicrons in 100 ml lymph.

TABLE 4

The amount of TG and PL (mg/100 ml) in chylomicron fractions and the mean volume ($Å^3$) and mean surface area ($Å^2$) of a random sample of 100 or 200 chylomicrons and Sf12-400 in thoracic duct lymph of rabbits fed a diet containing either 5 per cent or 30 per cent corn oil.

		Rabbit No.	TG mg/100 ml	PL mg/100 ml	Mean Volume $10^7 Å^3$	Mean Surface Area $10^5 Å^2$
	Chylomicrons	1	966	86	65	29
	"	2	482	47	59	30
	"	3	1090	121	109	47
5 per	"	4	207	23	39	23
cent	"	5	154	21	57	30
corn	Sf 12-400	1	68	17	-	-
oil	"	2	136	34	2.8	4.3
	"	3	113	30	9.4	9.6
	"	4	73	18	9.1	9.4
	"	5	70	17	-	-
	Chylomicrons	6	3170	210	180	61
	"	7	1960	116	217	68
	"	8	4140	313	189	64
	"	9	2250	182	187	60
30 per	"	10	4150	256	379	94
cent	Sf 12-400	6	112	20	-	-
corn	"	7	79	17	-	-
oil	"	8	144	37	6.4	7.5
	"	9	103	25	-	-
	"	10	54	15	10.4	12.7

Figure 18

The amount of triglyceride in the chylomicron and VLDL fractions of thoracic duct lymph of rabbits fed 5 per cent and 30 per cent corn oil

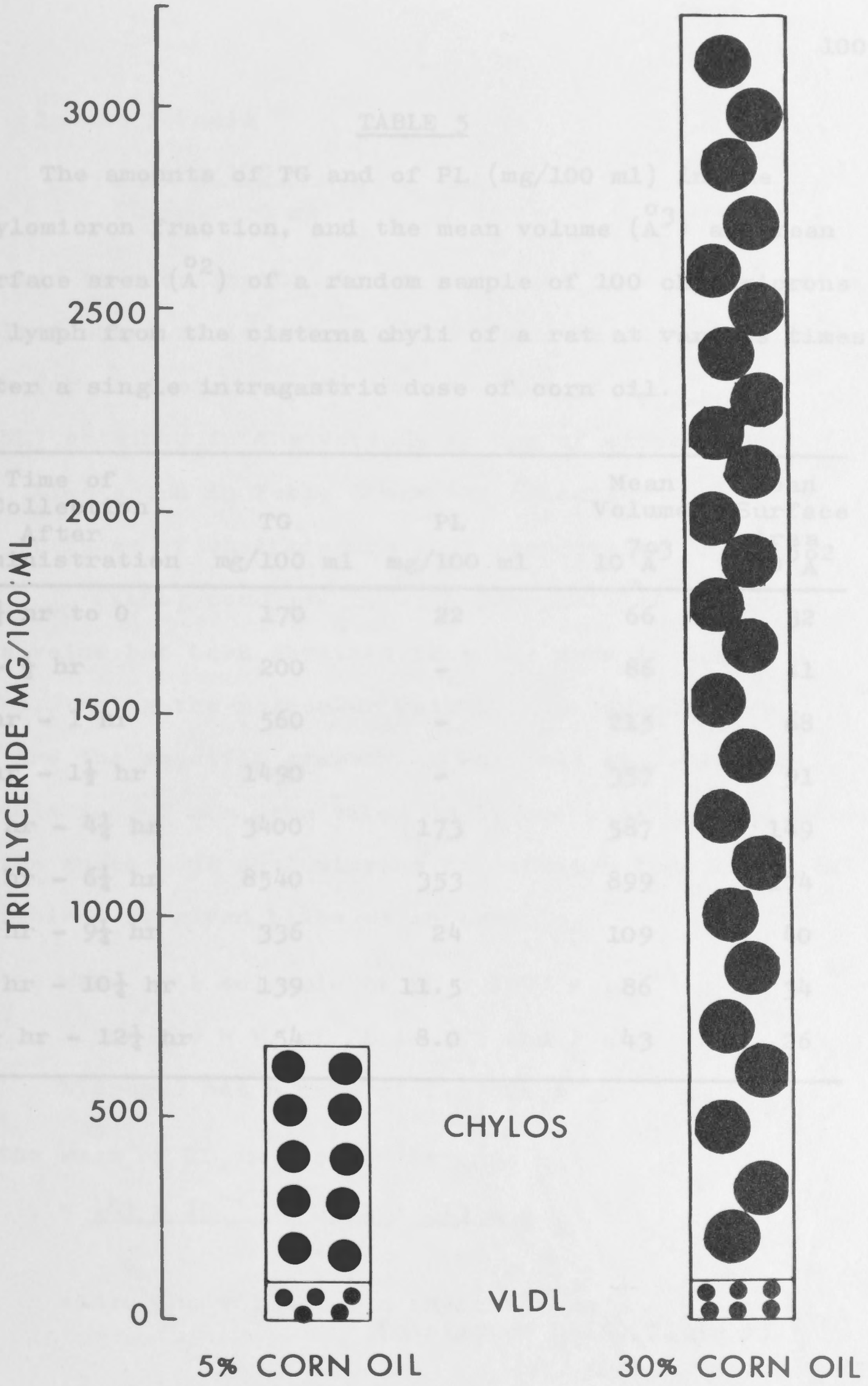

3000
2500
2000
1500
1000
500
0
TRIGLYCERIDE MG/100 ML
CHYLOS
VLDL
5% CORN OIL
30% CORN OIL

TABLE 5

The amounts of TG and of PL (mg/100 ml) in the chylomicron fraction, and the mean volume ($\mathring{A}^3$) and mean surface area ($\mathring{A}^2$) of a random sample of 100 chylomicrons in lymph from the cisterna chyli of a rat at various times after a single intragastric dose of corn oil.

Time of Collection After Administration	TG mg/100 ml	PL mg/100 ml	Mean Volume $10^7 \mathring{A}^3$	Mean Surface Area $10^5 \mathring{A}^2$
$-1\frac{3}{4}$ hr to 0	170	22	66	32
0 - $\frac{1}{2}$ hr	200	-	86	41
$\frac{1}{2}$ hr - 1 hr	560	-	215	68
1 hr - $1\frac{1}{2}$ hr	1490	-	357	91
$2\frac{1}{4}$ hr - $4\frac{1}{4}$ hr	3400	173	587	149
$4\frac{1}{4}$ hr - $6\frac{1}{4}$ hr	8540	353	899	174
$8\frac{1}{4}$ hr - $9\frac{1}{4}$ hr	336	24	109	40
$9\frac{1}{4}$ hr - $10\frac{1}{4}$ hr	139	11.5	86	34
$10\frac{1}{4}$ hr - $12\frac{1}{4}$ hr	54	8.0	43	26

In the formula

$$\frac{\text{TG, (mg/100 ml)}}{\text{PL, (mg/100 ml)}} = k \frac{\Sigma V}{\Sigma SA}$$

k would be a constant. The mean values of k are given in Table 6 where it is evident that there is little difference in k for chylomicrons and lipoproteins of $S_{f12-400}$ (VLDL) obtained in the various groups of experiments.

Also shown in Table 6 are the calculated areas on the surfaces of chylomicrons and lipoproteins of $S_{f12-400}$ (VLDL) occupied by each phospholipid molecule. This value has been obtained from the data in Tables 4 and 5, taking the molecular weight of phospholipid as 775 and the specific gravity of corn oil as 0.92 g/ml. The calculation of the area taken up by one phospholipid molecule on the surface of chylomicrons of rabbit 8 from the data in Table 4 is given below as an example.

A the mass of 1 molecule of PL = 1297×10^{-24} g

since the M.W. of PL is 775 and 1 atom of hydrogen has a mass of 1.67326×10^{-24} g

B the mass of PL in n chylomicrons

$$= \frac{189 \times 10^{-17} \times 0.92 \times 313 \times n}{4140} \text{ g}$$

since the volume of n chylomicrons

(continued below Table 6)

TABLE 6

The value of k from the equation $\frac{TG}{PL} = k \frac{\sum V}{\sum SA}$ and the area occupied by each PL molecule on the surface of lipid particles. The results are calculated from data in Tables 4 and 5 and expressed as mean and S.E. of mean from each group of animal samples.

Animal	Particles	Amount of Corn oil in diet	k x 10^4	Area per PL molecule $\mathring{A}^2$
Rabbit	Chylomicrons	5 per cent	465 ± 32	65 ± 4.5
	Chylomicrons	30 per cent	485 ± 27	64 ± 3.8
	$S_{f12-400}$(VLDL)	5 and 30 per cent	463 ± 40	65 ± 5.6
Rat	Chylomicrons	0.5 ml as single dose	457 ± 22	64 ± 3.1

(B continued)

$= 189 \times 10^7 \times n \mathring{A}^3$

$= 189 \times 10^{-17} \times n$ cc

since 1 $\mathring{A}^3 = 10^{-24}$ cc

and since the SG of corn oil is 0.92 g/ml

then mass TG in n chylos = $189 \times 10^{-17} \times n \times 0.92$ g

and since in this sample $\frac{PL}{TG} = \frac{313}{4140}$

then the mass of PL in n chylomicrons is equal to the equation B.

C the number of PL molecules in n chylomicrons

$$= \frac{B}{A}$$

$$= \frac{189 \times 10^{-17} \times 0.92 \times 313 \times n}{4140} \times \frac{1}{1297 \times 10^{-24}}$$

D and since the surface area of n chylomicrons

$$= 64\ n \times 10^{5}\ \text{A}^{\circ 2}$$

E then area taken up by 1 PL molecule

$$= \frac{D}{C}$$

$$= \frac{64n \times 10^{5} \times 1297 \times 10^{-24} \times 4140}{189 \times 10^{-17} \times 0.92 \times 313 \times n}$$

$$= 63\ \text{A}^{\circ 2}$$

Other values can be calculated from the data in Tables 4 and 5 from the formula given below: -

$$\text{Area taken up by one PL molecule} = \frac{\overline{SA} \times 1297 \times TG}{\overline{V} \times 92 \times PL}\ \text{A}^{\circ 2}$$

The concentrations of TG and PL were also determined in chylomicrons from lymph obtained from rabbits fed a diet containing 5 or 30 per cent butter. The $\frac{TG}{PL}$ ratio from these determinations as compared with similar figures from corn oil fed animals are shown in Table 7. It can be seen that the $\frac{TG}{PL}$ ratio of chylomicrons from both butter and corn oil fed animals is higher from the animals on the higher fat diet.

TABLE 7

The $\frac{TG}{PL}$ ratios in chylomicrons obtained from the thoracic duct lymph of rabbits fed a diet containing 5 per cent (5 animals) and 30 per cent (5 animals) of corn oil and 5 per cent (7 animals) and 30 per cent (6 animals) of butter. Results expressed as mean and S.E. of mean.

Dietary Fat	Corn Oil	Butter
5 per cent	9.4 ± 0.7	9.5 ± 0.8
30 per cent	14.8 ± 0.9	16.4 ± 1.0

B. THE SIZE AND COMPOSITION OF PARTICULATE LIPID IN VARIOUS ARTIFICIAL FAT EMULSIONS COMPARED WITH CHYLOMICRONS.

Another means by which particulate lipid may reach the blood stream is by the experimental, and more recently, the clinical use of intravenous injections of artificial fat emulsions.

The sizes of particulate lipid in artificial fat emulsions was determined by electron microscopy in a similar manner to that used for chyle. Random samples

of 200 particles were counted in each case. Figure 19 is a composite electron micrograph comparing osmium fixed particles. When shadowed with chromium these particles were shown to be spherical in shape.

The percentage distributions of diameters of particles from various experimental emulsions of batch number 140967 have been plotted in Fig. 20. Fig. 21 shows the distribution of diameters from the two commonly used commercial soya bean oil emulsions, Intralipid 10 and 20 per cent, and demonstrates the difference in size distribution between new and old samples.

A comparison of the sizes of particles from various artificial fat emulsions, the composition of which have been shown in Table 1, and of naturally occurring chylomicrons, is shown in Table 8. As in the case of chylomicrons when the phospholipid molecular area was calculated it was assumed that the triglyceride was carried in the core while the phospholipid was evenly spread over the surface of all the particles in the sample. The assumption was made from the similarity in electron microscopic appearance between sectioned chylomicrons and artificial lipid particles as shown by Schoefl (1968).

Figure 19

Composite electron micrographs at the same magnification of various artificial fat emulsions, and for comparison, chylomicrons from the chyle of a rabbit fed 30 per cent corn oil. The polystyrene marker is inserted as a scale.

(For composition of the artificial fat emulsions see Table 1, for measurement see Table 8)

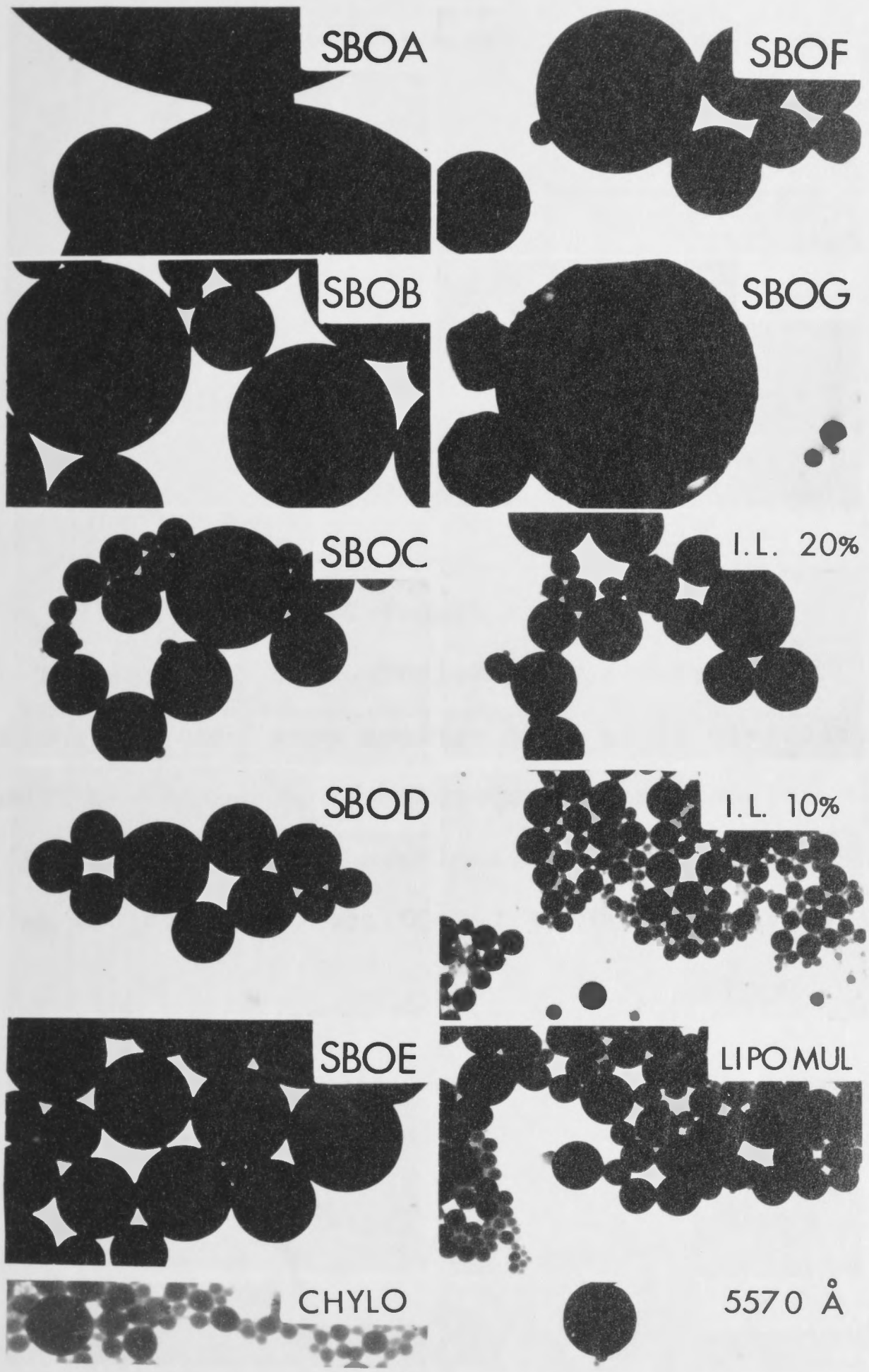

SBOA
SBOF
SBOB
SBOG
SBOC
I.L. 20%
SBOD
I.L. 10%
SBOE
LIPOMUL
CHYLO
5570 Å

Figure 20

The percentage distribution of diameters of particulate lipid from various soya bean oil emulsions with an increasing proportion of phospholipid from A to G. For comparison chylomicron diameter distribution from the chyle of a rabbit fed 30 per cent corn oil is depicted.

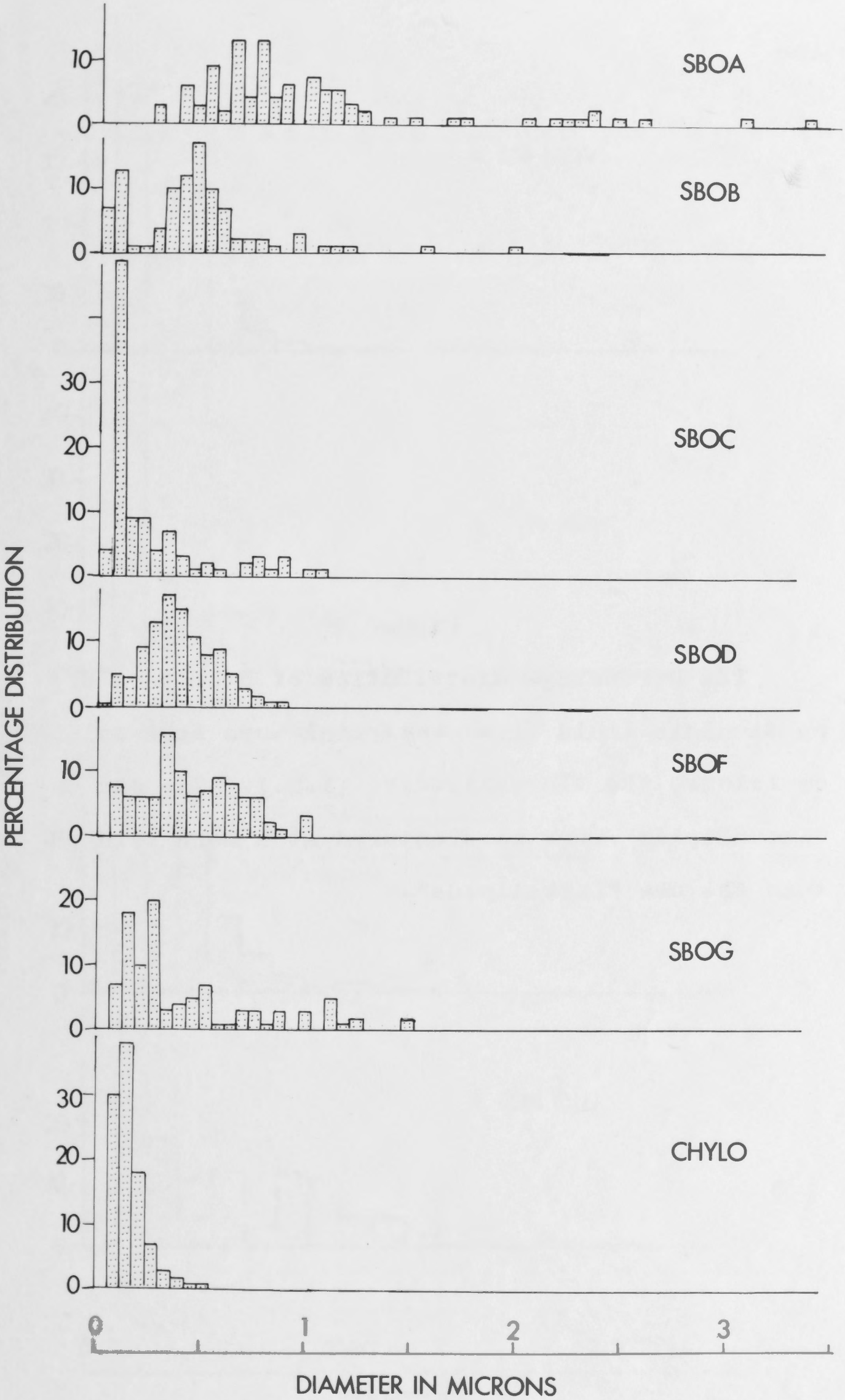

PERCENTAGE DISTRIBUTION
SBOA
SBOB
SBOC
SBOD
SBOF
SBOG
CHYLO
0
10
20
30
1
2
3
DIAMETER IN MICRONS

Figure 21

The percentage distribution of diameters of particulate lipid from commercial soya bean oil emulsions, the "Intralipids". (I.L.). The old "Intralipids" were manufactured at a more acid pH than the new "Intralipids".

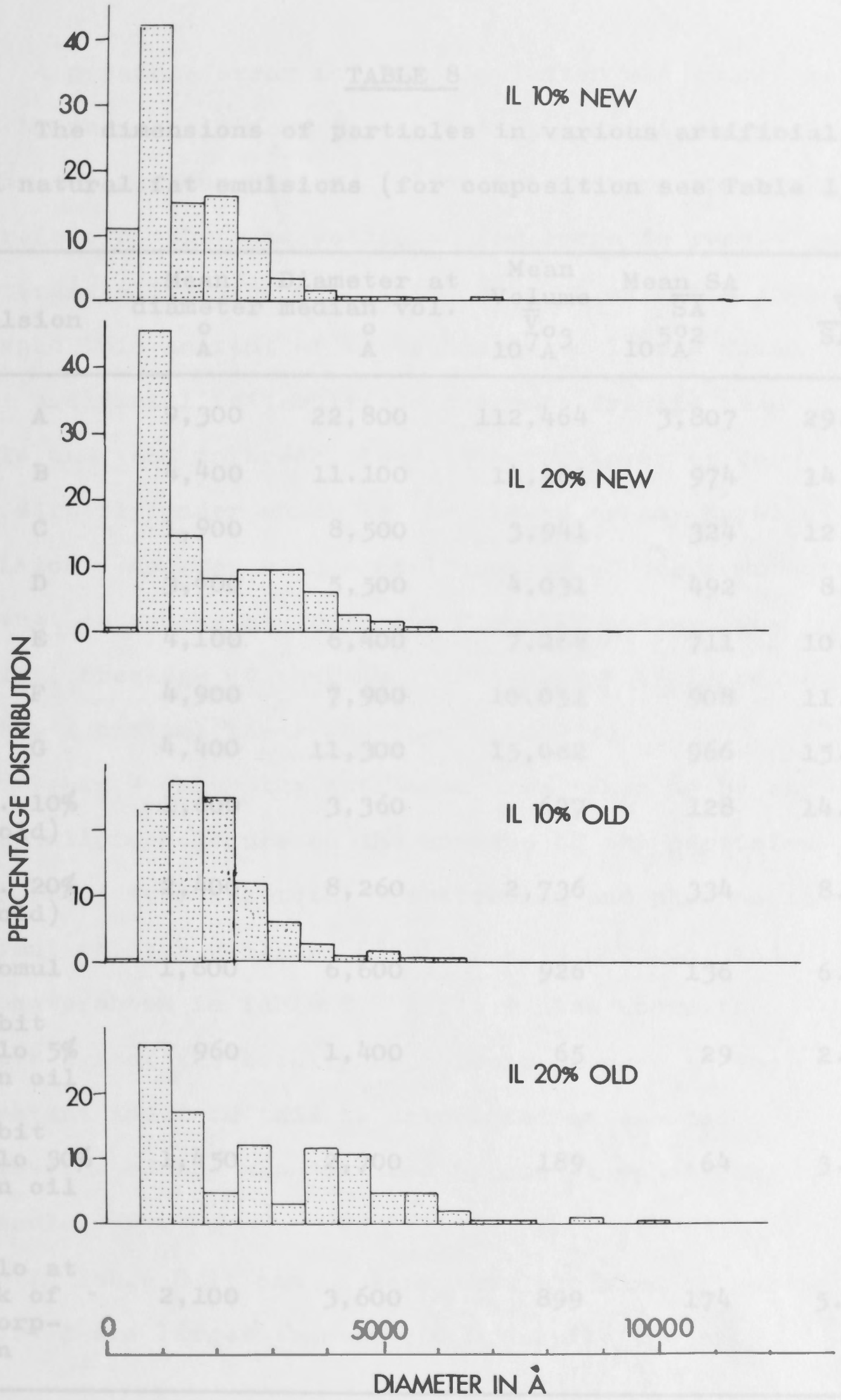

IL 10% NEW
IL 20% NEW
IL 10% OLD
IL 20% OLD
PERCENTAGE DISTRIBUTION
0
10
20
30
40
0
5000
10000
DIAMETER IN Å

TABLE 8

The dimensions of particles in various artificial and natural fat emulsions (for composition see Table 1)

Emulsion	Mean diameter Å	Diameter at median vol. Å	Mean Volume $\overline{V}$ $10^7 Å^3$	Mean SA $\overline{SA}$ $10^5 Å^2$	$\frac{\overline{V}}{SA}$
SBO A	9,300	22,800	112,464	3,807	29.5
SBO B	4,400	11.100	14,461	974	14.8
SBO C	1,900	8,500	3,941	324	12.2
SBO D	3,600	5,500	4,031	492	8.2
SBO E	4,100	6,400	7,288	711	10.2
SBO F	4,900	7,900	10,051	908	11.1
SBO G	4,400	11,300	15,082	966	15.6
I.L. 10% (old)	1,600	3,360	627	128	14.9
I.L. 20% (old)	2,400	8,260	2,736	334	8.2
Lipomul	1,800	6,600	926	136	6.8
Rabbit chylo 5% corn oil	960	1,400	65	29	2.2
Rabbit chylo 30% corn oil	1,450	2,700	189	64	3.0
Rat chylo at peak of absorption	2,100	3,600	899	174	5.2

A possible error in the calculation was that some of the phospholipid in the artificial emulsion might be in the aqueous phase. The emulsions were spun therefore by the same method as for lymph to remove the particulate lipid of $S_{f>400}$ in order to estimate the phospholipid content of the subnatant. It was found that artificial fat emulsions are more fragile than chyle and tend to break, leaving a top layer of corn oil directly under which is the packed creamy layer of emulsion. However with centrifugation of the same order as that to remove chylomicrons from lymph there was minimal breakage of emulsion. The phospholipid content of the subnatant layer was then estimated.

Table 9 shows the estimated area taken up by one phospholipid molecule on the surface of the particles calculated from the total triglyceride and phospholipid content of each emulsion (Table 1 - in methods) and the data shown in Table 8. Table 9 also shows the percentage of the total phospholipid present in the subnatant and from this is calculated an amended estimation of the area covered by one phospholipid molecule.

In Table 8 it can be seen that the size of particles in SBO E are larger than in SBO D despite the same

TABLE 9

The area taken up by one PL molecule on the surface of particles from various artificial fat emulsions calculated from the data in Tables 1 and 8.

Emulsion	Area covered by 1 PL molecule $\mathring{A}^2$	Percentage of PL in subnatant	Amended estimate of area of 1 PL molecule $\mathring{A}^2$
SBO A	63	7	67
SBO B	63	7	67
SBO C	38	5	40
SBO D	28	19	35
SBO E	23	15	27
SBO F	10	33	15
SBO G	4	42	7
I.L. 10% (old)	24	40	40
I.L. 20% (old)	29	24	38
Lipomul	26	43	46
Rabbit chylo 5% corn oil	70	-	-
Rabbit chylo 30% corn oil	63	-	-
Rat chylo at peak of absorption	65	-	-

$\frac{TG}{PL}$ ratio. One difference between these two lipid emulsions was the pH at which they were manufactured. From Table 1 it can be seen that SBO E was more acid than SBO D.

To test the reproducibility of this finding other artificial fat emulsions were prepared by the manufacturer at the same $\frac{TG}{PL}$ ratio but at different pH. These were then examined by electron microscopy for diameter distribution. Fig. 22 is a composite electron micrograph of SBO D, SBO E and SBO H from batch 150868 manufactured with the same $\frac{TG}{PL}$ ratios but at varying pH. Fig. 23 is a diagram showing diameter distributions of these emulsions. It can be seen that the particles of more acid emulsion are on the whole larger. The particles in SBO I were also found to be larger than the particles in SBO K (Fig. 24) as were the particles of old Intralipid as compared with new Intralipid (Fig. 21).

The sterol content of artificial fat emulsions was also determined since it seemed possible that some toxic phenomena related to their intravenous administration might be due to phytosterols entering the blood stream. Table 10 shows the sterol content of some of the soya bean oil emulsions with differing TG (vegetable source) and PL (egg source) concentrations. Also included are results from Lipomul in which both the TG and PL are derived from vegetable source.

Figure 22

Composite electron micrographs of soya bean oil emulsions manufactured with the same $\frac{TG}{PL}$ ratio but at varying pH.

The larger particles of SBO E were manufactured at a more acid pH than SBO D which in turn was more acid than SBO H (see Table 1).

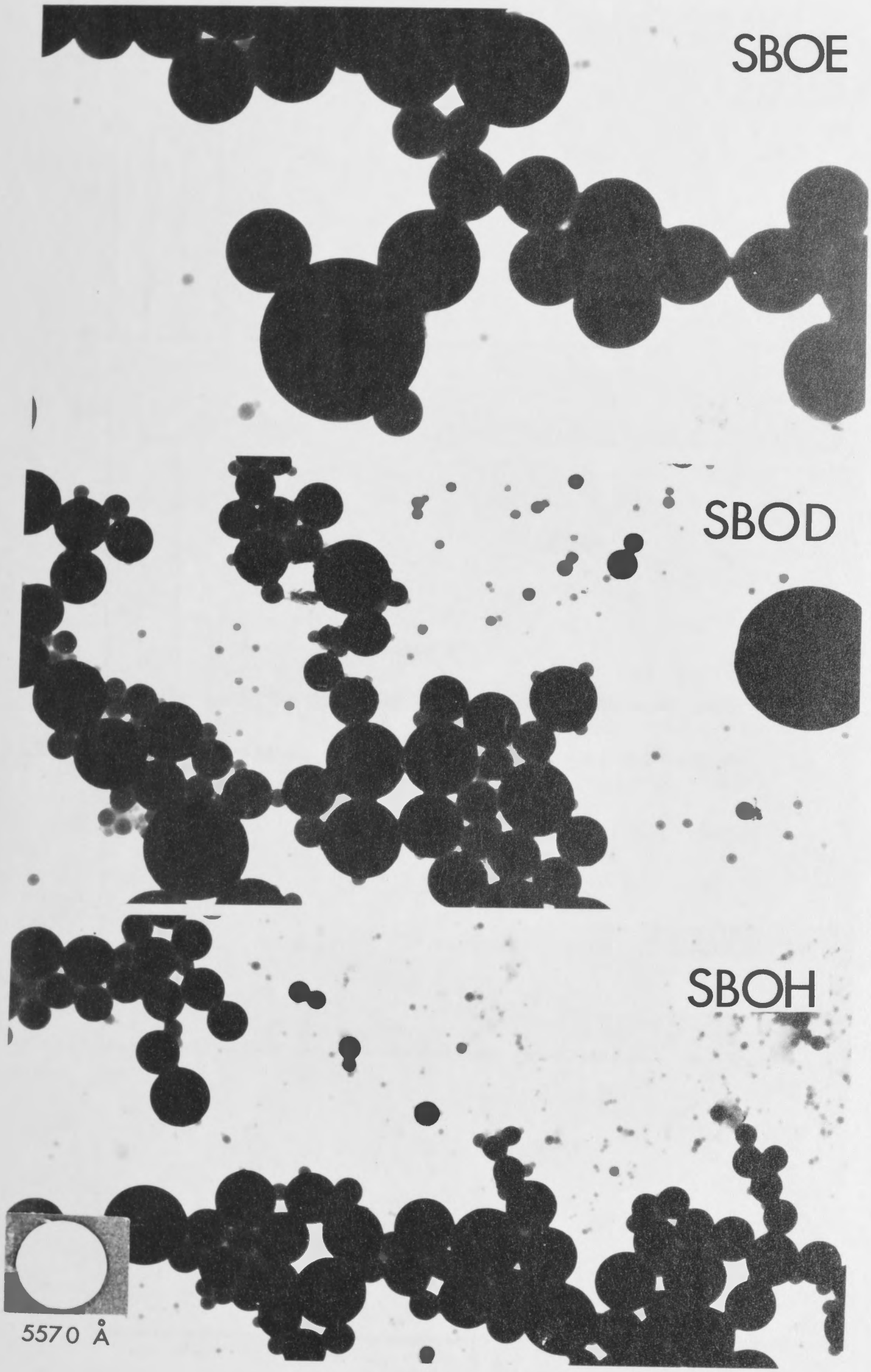

SBOE
SBOD
SBOH
5570 Å

Figure 23

The percentage distribution of the diameters of the particles from emulsions depicted in Fig. 22.

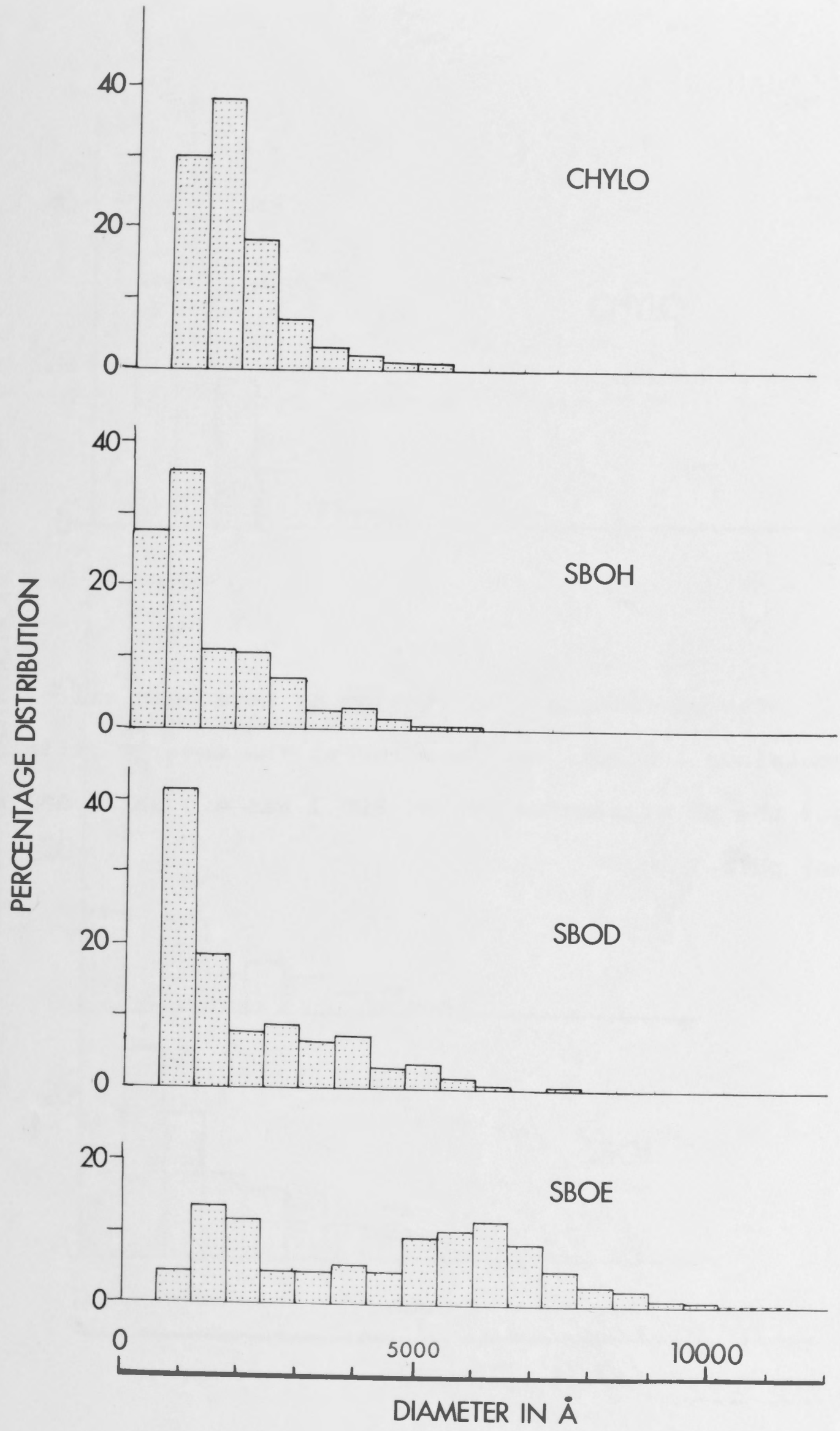

PERCENTAGE DISTRIBUTION
40
20
0
CHYLO
40
20
0
SBOH
40
20
0
SBOD
20
0
SBOE
0
5000
10000
DIAMETER IN Å

Figure 24

The percentage distribution of soya bean oil emulsions I and K, manufactured at the same $\frac{TG}{PL}$ ratio but the pH at manufacture of SBO I was 6.0 while SBO K was pH 6.5

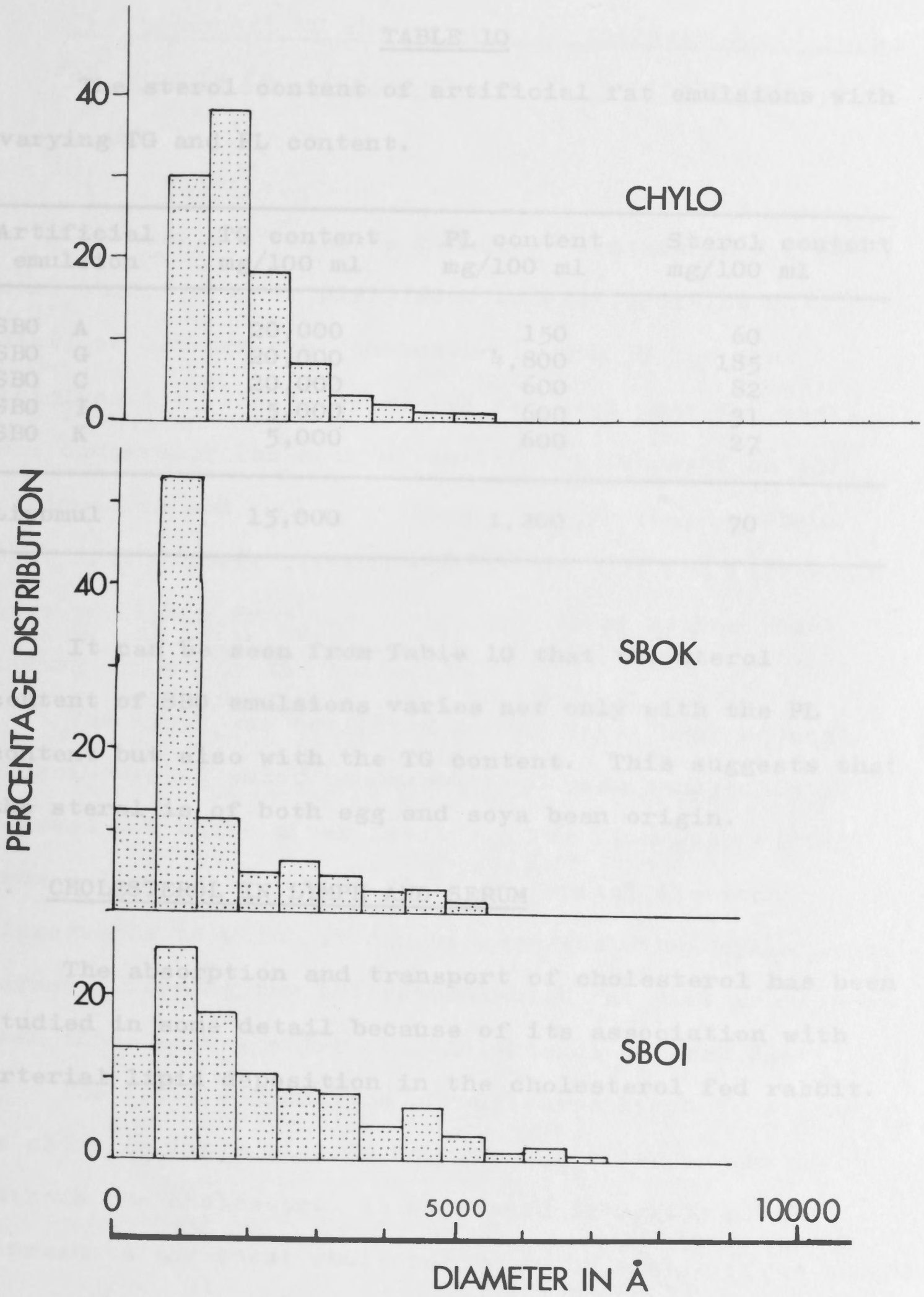

40
20
0
CHYLO
PERCENTAGE DISTRIBUTION
40
20
SBOK
20
0
SBOI
0
5000
10000
DIAMETER IN Å

TABLE 10

The sterol content of artificial fat emulsions with varying TG and PL content.

Artificial emulsion	TG content mg/100 ml	PL content mg/100 ml	Sterol content mg/100 ml
SBO A	20,000	150	60
SBO G	20,000	4,800	185
SBO C	20,000	600	82
SBO I	5,000	600	31
SBO K	5,000	600	27
Lipomul	15,000	1,200	70

It can be seen from Table 10 that the sterol content of SBO emulsions varies not only with the PL content but also with the TG content. This suggests that the sterol is of both egg and soya bean origin.

C. CHOLESTEROL IN LYMPH AND SERUM

The absorption and transport of cholesterol has been studied in some detail because of its association with arterial lipid deposition in the cholesterol fed rabbit.

1. The transport of cholesterol in thoracic duct lymph of animals fed cholesterol with varying triglyceride loads

(i) Rabbits

The chyle of rabbits fed diets containing 0.8 per cent cholesterol in plain food (cholesterol-low fat diet) or 0.8 per cent cholesterol with 30 per cent triglyceride (cholesterol-high fat diet) for 4-8 weeks was compared. The main attention was focussed on the chylomicron and VLDL fractions of lymph since probably most of the small lipoproteins of D>1.019 are filtered into the lymph from the peripheral serum rather than having origin in the intestine.

The lymph was collected in the first hour of acute experiments in which the rabbit had been cannulated at between 4-6 hours after feeding. The flow rate varied from 5-10 ml/hour. Fig. 25 is of typical electron micrographs in which it can be seen that the chylomicrons formed following the cholesterol-high fat diet are larger than those formed after the cholesterol-low fat diet.

The lipid composition of the three lymph fractions is shown in Table 11. As described earlier in the Methods the cholesterol is expressed in mg/100 ml and represents the total cholesterol of the sample (free and

Figure 25

Composite electron micrographs at the same magnification of chylomicrons and VLDL from a rabbit fed cholesterol-low fat diet (A), and cholesterol-30 per cent corn oil (B).

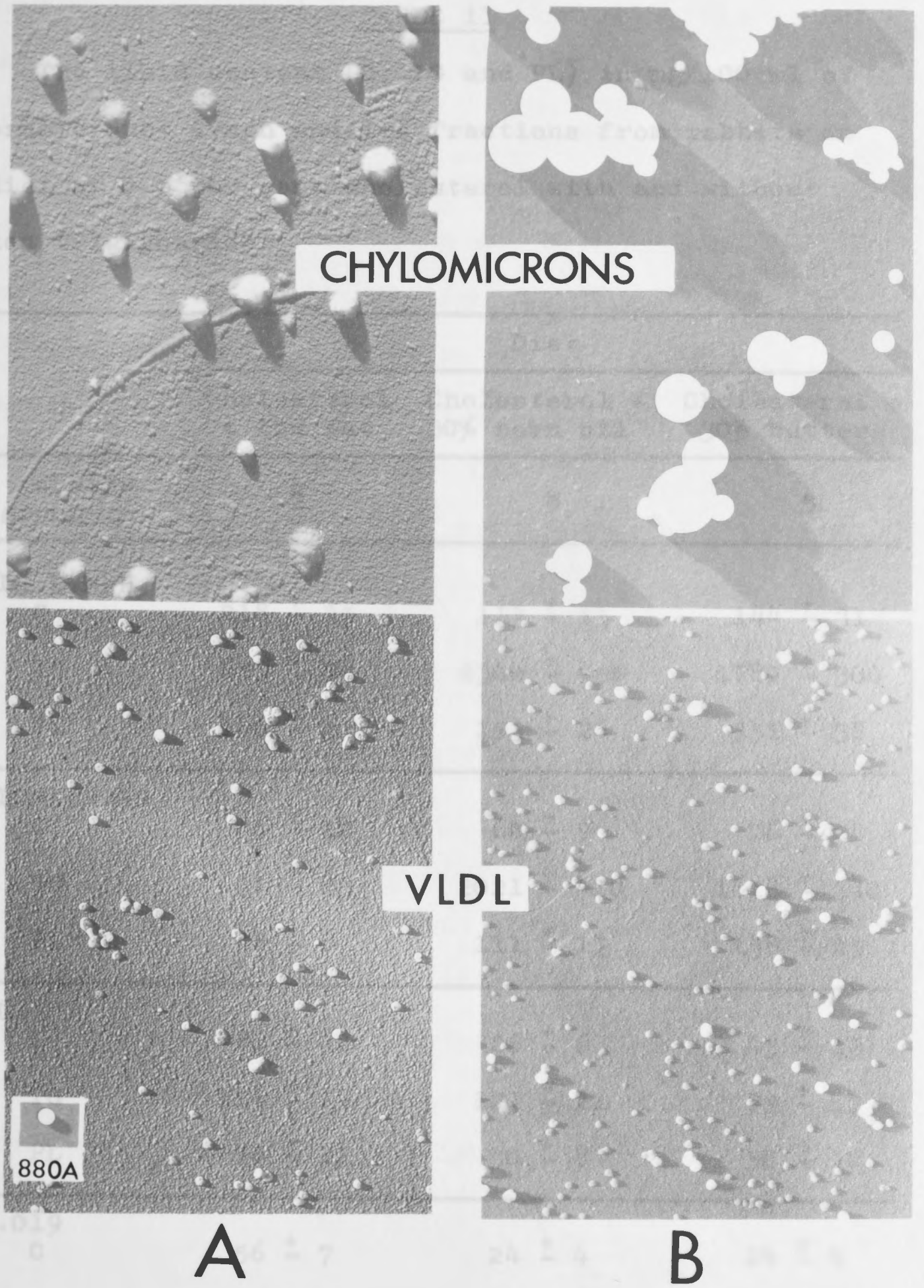

CHYLOMICRONS
VLDL
880A
A
B

TABLE 11

The lipid content (C, TG and PL) in mg/100 ml of thoracic duct lymph and its fractions from rabbits on a diet of 0.8 per cent cholesterol with and without added triglyceride

	Diet		
	Cholesterol + low fat	Cholesterol + 30% corn oil	Cholesterol + 30% butter
No. rabbits in group	8	8	3
WHOLE LYMPH			
C	218 ± 35	113 ± 15	144 ± 31
TG	653 ± 94	2301 ± 450	1780 ± 300
PL	160 ± 22	193 ± 24	351 ± 38
CHYLOMICRONS			
C	66 ± 12	60 ± 9	76 ± 24
TG	474 ± 83	2021 ± 410	1618 ± 340
PL	48 ± 8	111 ± 17	138 ± 25
VLDL			
C	81 ± 10	26 ± 6	43 ± 15
TG	124 ± 24	121 ± 21	99 ± 17
PL	56 ± 11	30 ± 8	50 ± 12
D>1.019			
C	56 ± 7	24 ± 4	24 ± 4
TG	23 ± 4	32 ± 6	32 ± 5
PL	54 ± 5	44 ± 7	57 ± 11

esterified) measured as free cholesterol. The lipid composition of the lymph has been diagramatically represented in Fig. 26. It can be seen that triglyceride is the major lipid in the chylomicron and VLDL fractions, especially in the chylomicrons from the rabbits on cholesterol-high fat diets.

The diameters of a random 600 particles were measured from the chylomicron and VLDL fractions of two groups of three rabbits. The first group was fed cholesterol and low fat diet and the second group cholesterol and 30 per cent corn oil. The mean diameter of the chylomicron fractions were 1065 and 1476 $\mathring{A}$ and those of the VLDL were 499 and 493 $\mathring{A}$ respectively. A diagram of the percentage distribution of diameters is shown in Fig. 27.

Fig. 28 represents the proportion of cholesterol found in the different fractions of thoracic duct lymph. It can be seen from Figs. 27 and 28 that relatively more cholesterol entering the blood stream from thoracic duct lymph is in larger lipid particles after a diet containing cholesterol plus high fat diet than after cholesterol plus low fat diet.

Figure 26

The lipid composition of the three fractions of thoracic duct lymph from rabbits fed 0.8% cholesterol with varying triglyceride loads. The data was obtained from Table 11.

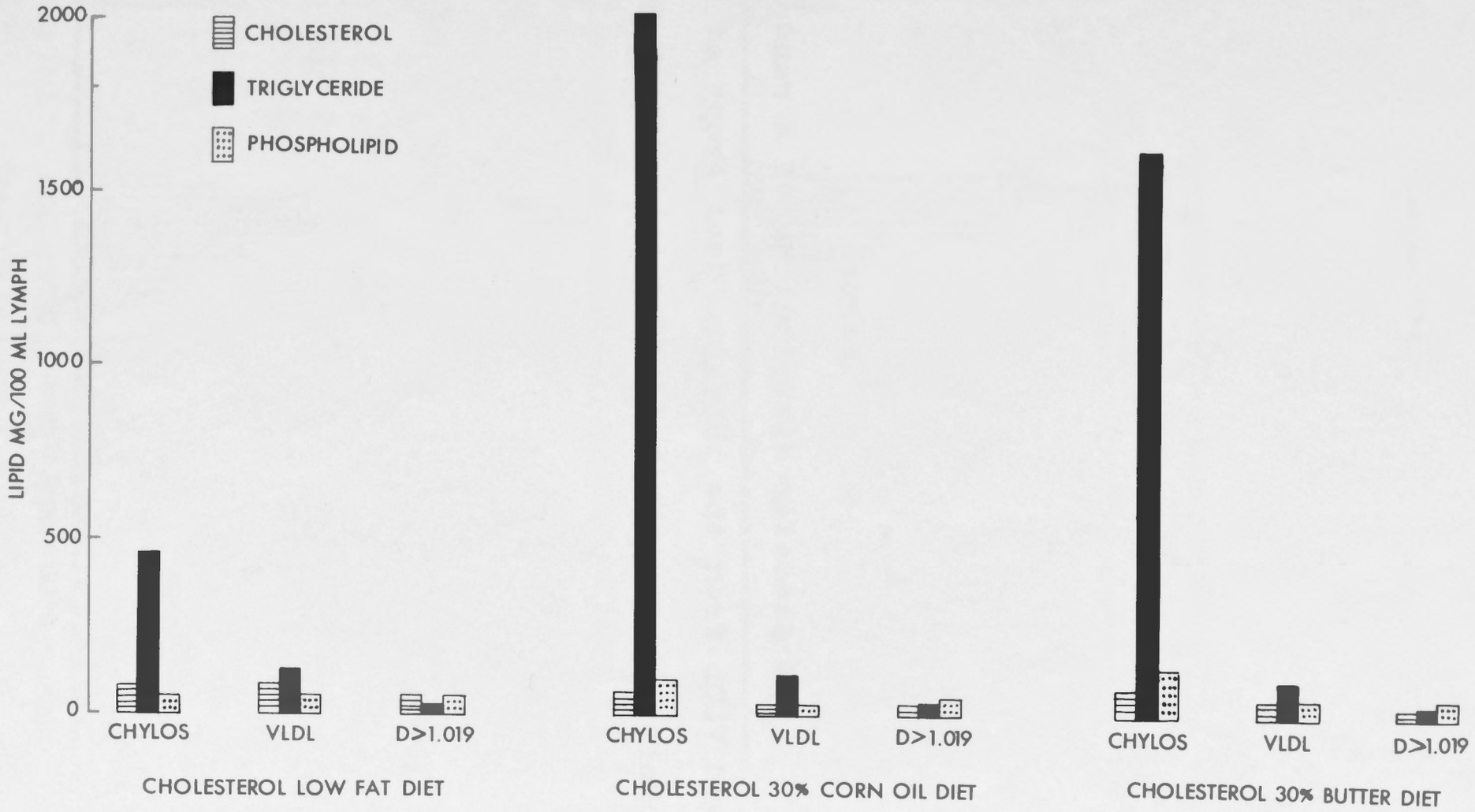

CHOLESTEROL
TRIGLYCERIDE
PHOSPHOLIPID
LIPID MG/100 ML LYMPH
2000
1500
1000
500
0
CHYLOS
VLDL
D>1.019
CHOLESTEROL LOW FAT DIET
CHOLESTEROL 30% CORN OIL DIET
CHOLESTEROL 30% BUTTER DIET

Figure 27

The diameter distribution of a random 600 chylomicrons and VLDL from the thoracic duct lymph of rabbits fed 0.8% cholesterol with plain food (A) or with 30% corn oil (B).

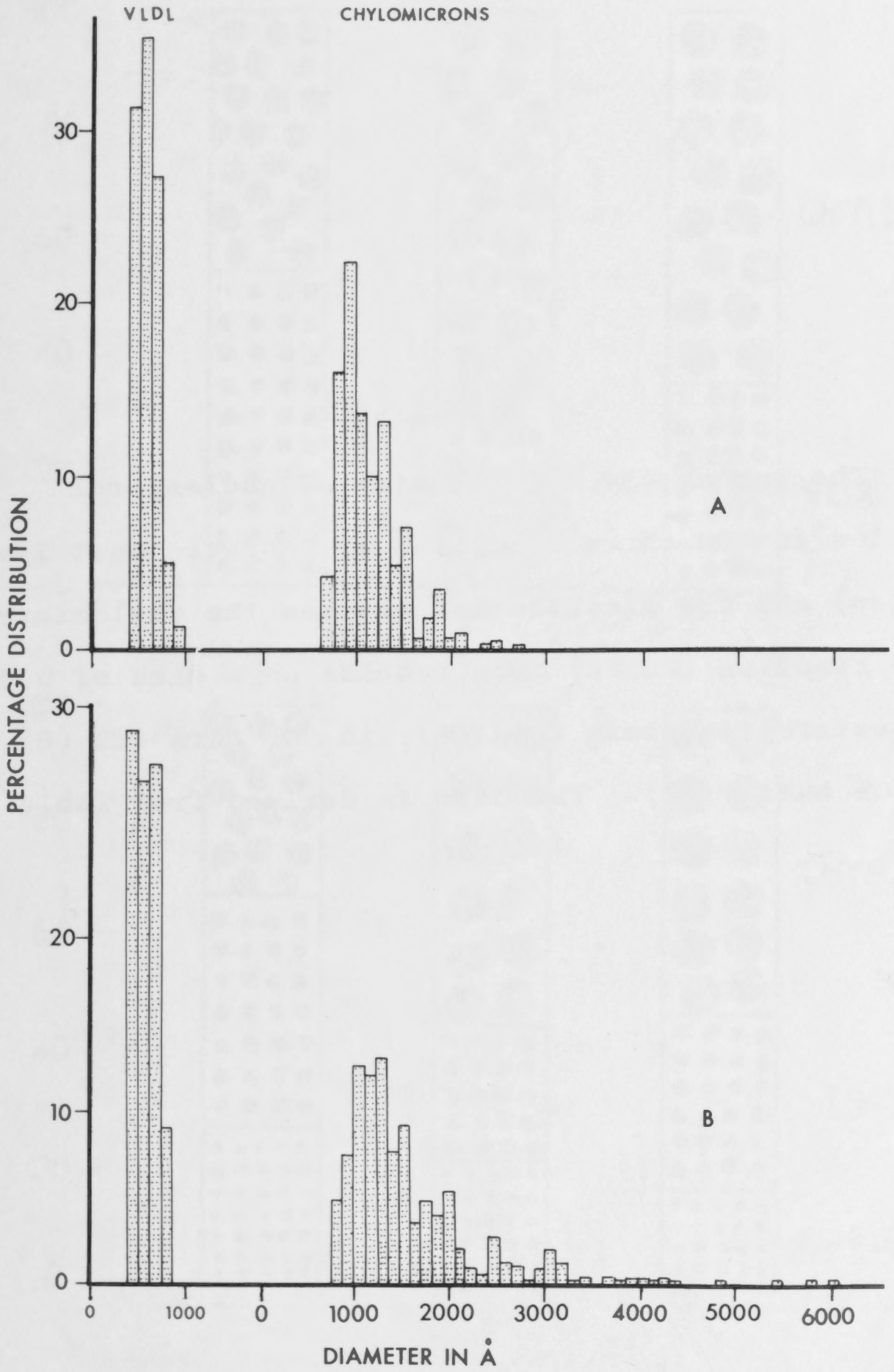
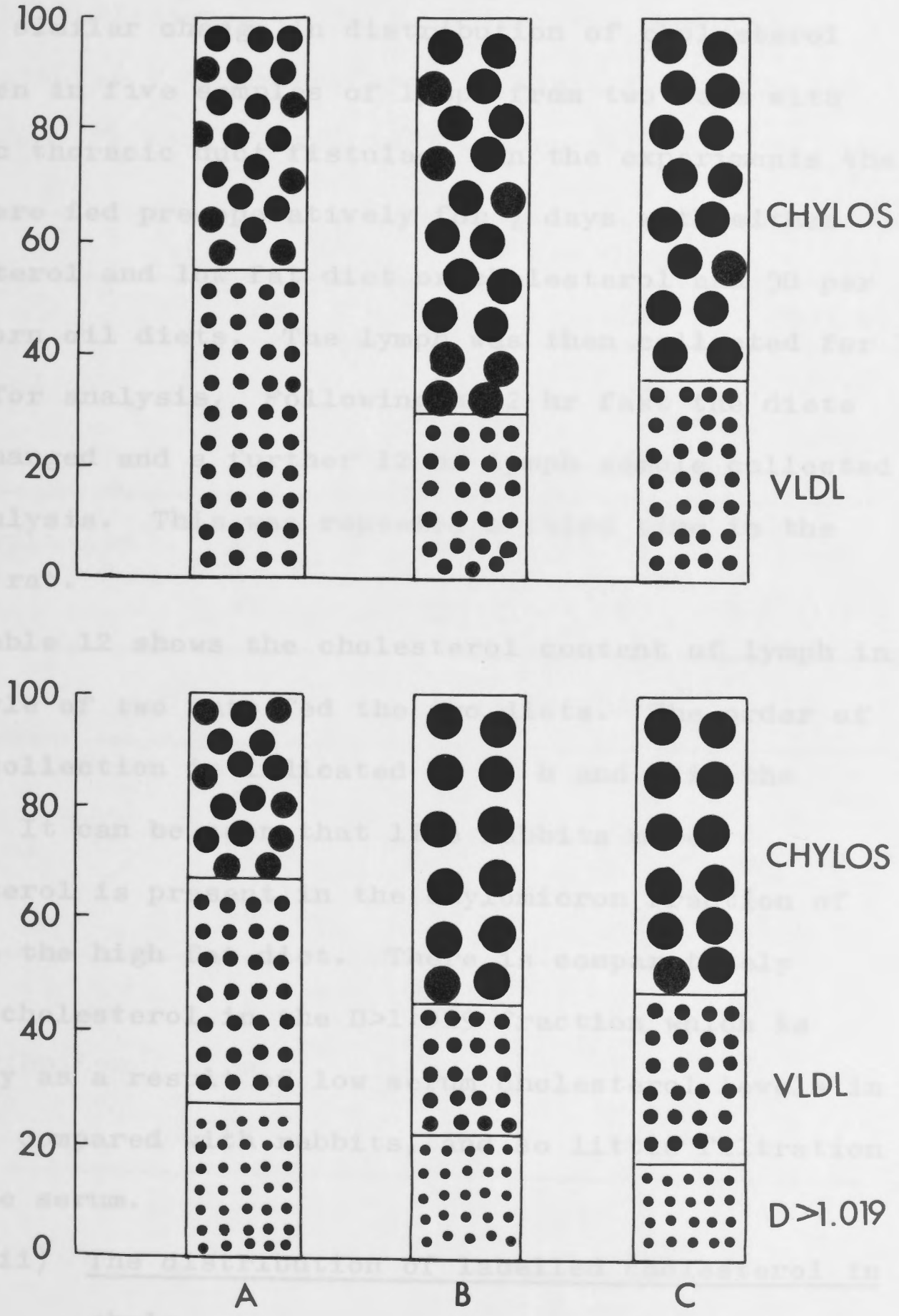

VLDL
CHYLOMICRONS
A
B
PERCENTAGE DISTRIBUTION
30
20
10
0
0
1000
0
1000
2000
3000
4000
5000
6000
DIAMETER IN Å

Figure 28

The percentage distribution of cholesterol carried in the three fractions of thoracic duct lymph (below) and the distribution between the chylomicron and VLDL fraction (above) from rabbits on a diet of 0.8%, cholesterol in plain food (A), in 30% corn oil (B), and in 30% butter (C). The data is derived from Table 2.


100
80
60
40
20
0
CHYLOS
VLDL
D>1.019
A
B
C

(ii) Rats

A similar change in distribution of cholesterol was seen in five samples of lymph from two rats with chronic thoracic duct fistulae. In the experiments the rats were fed pre-operatively for 7 days with either cholesterol and low fat diet or cholesterol and 30 per cent corn oil diets. The lymph was then collected for 12 hr for analysis. Following a 12 hr fast the diets were changed and a further 12 hr lymph sample collected for analysis. This was repeated a third time in the second rat.

Table 12 shows the cholesterol content of lymph in the chyle of two rats fed the two diets. The order of lymph collection is indicated by a, b and c in the table. It can be seen that like rabbits more cholesterol is present in the chylomicron fraction of rats on the high fat diet. There is comparatively little cholesterol in the D>1.019 fraction which is possibly as a result of low serum cholesterol levels in rats as compared with rabbits, and so little filtration from the serum.

(iii) The distribution of labelled cholesterol in chyle

The distribution of labelled cholesterol within the thoracic duct lymph of rabbits fed cholesterol and

TABLE 12

The cholesterol content of thoracic duct lymph from rats fed 0.8% cholesterol in plain food (low fat diet) or 0.8% cholesterol in 30% corn oil (high fat diet)

		Low Fat Diet		
	Rat	Chylo	VLDL	D>1.019
mg/100 ml	1a	24	26	11
	2b	14	25	3
% distribution	1a	39	43	18
	2b	33	60	7
		High Fat Diet		
	1b	42	8	4
mg/100 ml	2a	190	30	10
	2c	68	19	1
	1b	78	15	7
% distribution	2a	82	14	4
	2c	77	22	1

low fat diets or cholesterol and 30 per cent corn oil was examined in rabbits with chronic thoracic duct fistulae produced 5 hr after feeding. The lymph was collected for 12 hr and pooled and in the case of rabbit 6 a second 12 hr specimen was then collected.

Table 13 shows the radioactivity and cholesterol content of these various fractions. It can be seen that in all cases there is more radioactivity in the chylomicron fraction, except in the one and only second 12 hr specimen from rabbit 6 in which more cholesterol is in the VLDL fraction. There also appears to be relatively more radioactivity in the chylomicron fraction from rabbits on the high fat than on the low fat diet. In the second specimen of 6 the triglyceride absorption had almost ceased, the triglyceride content having fallen from 2,360 mg/100 ml to 440 mg/100 ml.

The percentage distribution of cholesterol, estimated both by radioactivity and chemically, is shown in Table 14. It is apparent that a greater proportion of cholesterol when estimated by the radioactivity, and thus representing ingested cholesterol, is in the chylomicron fraction than as suggested by chemical estimation. The reasons for this discrepancy

TABLE 13

The amount of radioactivity (R in counts/min/ml) and cholesterol concentration (C in mg/100 ml) in the thoracic duct lymph from rabbits fed ^{14}C-cholesterol during various diets containing cholesterol.

Fraction of chyle	Cholesterol + low fat diet						Cholesterol + 30% corn oil diet						Late absorption	
Rabbit No.	1		2		3		4		5		6(1)		6(2)	
	R	C	R	C	R	C	R	C	R	C	R	C	R	C
Chylo	1600	88	6250	50	950	65	24000	55	4120	60	275	26	100	33
VLDL	1050	111	2500	57	360	55	3000	20	900	22	0	12	130	72
D>1.019	500	52	600	48	180	35	150	33	100	22	0	9	80	64
Whole lymph	3250	250	8825	175	1600	156	27000	106	3900	99	275	153	300	200

TABLE 14

The percentage distribution of cholesterol, measured chemically and by radioactivity, in the thoracic duct lymph of rabbits fed cholesterol-low fat and cholesterol + 30% corn oil diets. (data obtained from Table 13).

	Rabbit No.	Counts			Total Cholesterol		
		Chylos	VLDL	D>1.019	Chylos	VLDL	D>1.019
Cholesterol Low Fat	1	51	33	16	35	44	21
	2	67	27	6	32	37	31
	3	64	24	12	42	36	23
Cholesterol + 30% Corn Oil	4	88	11	1	51	19	31
	5	80	18	2	58	21	21
	6(1)	100	0	0	55	25	20
Late Absorption	6(2)	32	43	25	19	43	38

will be discussed in the next chapter but include the possibility of cholesterol filtered from the serum in the D>1.019 and VLDL fraction, cholesterol synthesised in the intestine or liver appearing in these two fractions, or ingested cholesterol from late absorption appearing in the VLDL or D>1.019 fraction as suggested by rabbit 6(2).

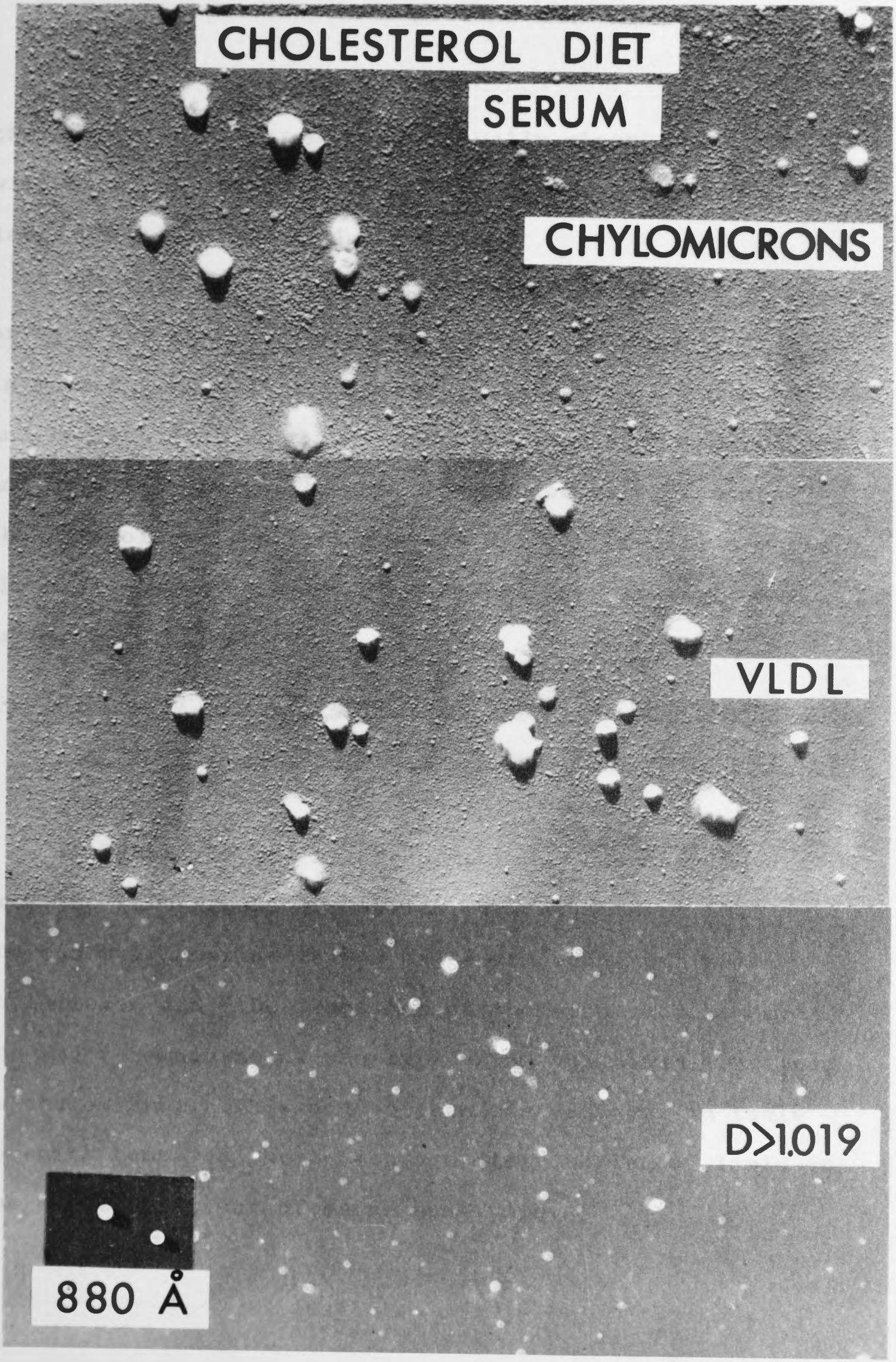
2. <u>The distribution of cholesterol present in serum of rabbits fed cholesterol with varying triglyceride loads</u>

The serum of rabbits fed cholesterol diets as described in the previous section was collected, separated into the three ultracentrifugal fractions and analysed.

Fig. 10 is of electron micrographs of lipoproteins from the thoracic duct lymph of a rabbit fed cholesterol-low fat diet and Fig. 29 is from the serum of the same animal for comparison. It can be seen that there is a similar pattern of distribution of the lipoprotein sizes, but that the particles are poorly fixed in serum and unsuitable for accurate measurement. However the VLDL of serum do appear larger in diameter than in lymph with diameters ranging from 360 - 1440 $\mathring{A}$. The serum of most of the rabbits fed cholesterol-low fat diet was rather turbid (whereas the serum from the animals on the

Figure 29

Electron micrographs at the same magnification of the chylomicron, VLDL and D>1.019 fractions of the hypercholesterolaemic serum from a rabbit fed cholesterol and plain food.


CHOLESTEROL DIET
SERUM
CHYLOMICRONS
VLDL
D>1.019
880 Å

cholesterol-high fat diet was usually clear) which, as will be discussed later, might be due to the comparatively large size of serum VLDL.

The results of the chemical analysis of the samples of sera are shown in Table 15. It can be seen that there is more cholesterol in the serum of rabbits fed cholesterol and low fat diet than in those rabbits fed cholesterol and high fat diet. The fractions which carry the most cholesterol are the VLDL and the D>1.019 fractions while the chylomicron fraction is comparatively small in all groups of rabbits.

3. <u>The comparison of the lipid content of the lipoproteins from thoracic duct lymph and serum of cholesterol fed rabbits</u>

If Tables 11 and 15 are compared, which are both from the same groups of rabbits, it can be seen that whereas triglyceride is the major component of the chylomicron and VLDL fractions of thoracic duct lymph, the major component of the lipid from the serum fractions is cholesterol. This can be seen more clearly in Table 16 which compares the percentage distribution of lipid in the three fractions of serum and lymph.

TABLE 15

The lipid content (C, TG and PL) in mg/100 ml of serum and its fractions from rabbits on a diet of 0.8 per cent cholesterol with and without added triglyceride

	Diet		
	Cholesterol + low fat	Cholesterol + 30% corn oil	Cholesterol + 30% butter
No. rabbits in group	8	8	3
Whole lymph			
C	1239 ± 278	350 ± 62	365 ± 118
TG	191 ± 67	140 ± 27	108 ± 22
PL	453 ± 82	161 ± 23	158 ± 24
Chylomicrons			
C	98 ± 56	50 ± 21	41 ± 22
TG	30 ± 11	30 ± 9	23 ± 9
PL	17 ± 3	9 ± 2	11 ± 5
VLDL			
C	602 ± 129	124 ± 40	189 ± 90
TG	95 ± 39	47 ± 11	44 ± 16
PL	212 ± 40	53 ± 16	67 ± 31
D>1.019			
C	407 ± 77	134 ± 27	67 ± 27
TG	60 ± 29	44 ± 10	24 ± 4
PL	227 ± 38	98 ± 21	67 ± 21

TABLE 16

The percentage composition of the various lipoprotein fractions from thoracic duct lymph and serum of cholesterol fed rabbits.

	Cholesterol + 30% corn oil		Cholesterol + low fat		Cholesterol + 30% butter	
	Lymph	Serum	Lymph	Serum	Lymph	Serum
Whole lymph or serum						
C	4	54	21	66	7	58
TG	88	21	63	10	82	17
PL	8	25	16	24	11	25
Chylomicrons						
C	3	56	11	67	4	55
TG	92	34	81	21	88	31
PL	5	10	8	12	8	14
VLDL						
C	15	55	31	66	22	63
TG	68	21	47	11	52	15
PL	17	24	22	23	26	22
D>1.019						
C	24	48	42	58	21	42
TG	32	16	17	9	28	15
PL	44	36	41	33	51	43

D. SOME ASPECTS OF THE FATE OF LIPOPROTEINS AFTER ENTRY INTO THE BLOOD STREAM

From the last section it can be seen that whereas lipoproteins enter the blood stream from the chyle of rabbits mainly as triglyceride rich chylomicrons, the predominant lipoproteins found in the serum of hypercholesterolaemic rabbits are smaller VLDL or D>1.019 particles with comparatively less triglyceride and more cholesterol. This section deals with experiments designed to attempt to explain this phenomenon and to follow some aspects of the fate and metabolism of the lipoproteins.

1. The action of clearing factor lipase on thoracic duct lymph chylomicrons in vitro

The enzyme CF lipase is thought to play a major role in the removal of TG from the blood stream. (Dole and Hamlin 1962). It has been shown that both *in vivo* and *in vitro* this enzyme will remove triglyceride from chylomicrons leaving smaller particles more rich in cholesterol (see discussion).

The results of incubating chylomicrons with post-heparin rabbit serum containing clearing factor (CF serum) and normal rabbit serum (control) are as follows:-

(i) Optical Density

The optical density of the non fractionated control samples remained about the same whereas the optical density of the sample in the clearing factor samples descrease by about 50%.

The optical densities of the different fractions separated after incubation by ultracentrifugation also showed a variation between the control and clearing factor. Two typical results are shown in Table 17. There was a decrease in the optical density of the chylomicron fraction and an increase in the optical densities of the other fractions in the clearing factor samples.

TABLE 17

Comparative optical densities (OD_{560}) from similar ultracentrifugal fractions after incubation of chylomicrons from rabbits 171 and 172 with normal and CF serum.

Fraction	Control		CF serum	
	R171	R172	R171	R172
Chylomicron	1.800	2.100	.800	1.650
Sub 1	.005	.020	.080	.020
Sub 2	.054	.096	.112	.170
VLDL	.055	.047	.205	.180
D>1.019	.080	.165	.225	.225

(ii) Radioactivity in the different fractions

The radioactive counts in each fraction of each experiment were also measured. Table 18 shows the distribution of counts (expressed as a percentage of the total number of counts and showing the percentage change) from the chylomicrons labelled with ^{3}H-palmitic acid and incubated with control and CF serum. The change here in the CF compared with the control is the decrease in palmitic acid in the chylomicron fraction and the increase in the palmitic acid in the D>1.019 fraction.

Table 19 shows the changes in cholesterol distribution as the chylomicrons here were labelled with ^{14}C-cholesterol. Again a decrease occurs in cholesterol in the chylomicrons incubated with clearing factor serum but in this case most of the cholesterol moved to the VLDL fraction rather than the D>1.019 fraction.

(iii) Chemical composition of chylomicron fractions

The thoracic duct lymph chylomicrons from four rabbits fed cholesterol - 30 per cent corn oil were analysed for triglyceride and cholesterol. The chylomicrons were then incubated with clearing factor or control serum for varying times at varying temperatures;

TABLE 18

The radioactive counts in various fractions, expressed as percentage of the total number of counts in each sample, together with the percentage change, after chylomicrons labelled with ^{3}H-palmitic acid have been incubated with normal serum (control) and post heparin serum (CF).

FRACTION	RABBIT 161			RABBIT 167			RABBIT 172		
	Control	CF	% change	Control	CF	% change	Control	CF	% change
Chylomicrons	90	64	-26	85	26	-59	91	43	-48
Subant-ant 1)	2	7	+ 5	2	5	+ 3	1	2	+ 1
Subnat-ant 2)				3	22	+19	1	14	+13
VLDL	6	7	+ 1	8	10	+ 2	4	12	+ 8
D>1.019	2	22	+20	2	37	+35	3	29	+26
Total no. of counts per 30 min.	52,863	41,153		17,554	16,910		85,812	69,281	

TABLE 19

The radioactive counts in various fractions, expressed as the percentage of the total number of counts in each sample, together with the percentage change, after chylomicrons labelled with ^{14}C-cholesterol have been incubated with normal serum (control) and post heparin serum (CF).

FRACTION	RABBIT 169			RABBIT 171			RABBIT 172		
	Control	CF	% change	Control	CF	% change	Control	CF	% change
Chylo-microns	52	18	-34	74	46	-28	67	44	-23
Subnat-ant 1	6	14	+ 8	2	8	+ 6	4	4	0
Subnat-ant 2	11	13	+ 2	5	7	+ 2	6	13	+ 7
VLDL	9	33	+24	6	22	+16	11	19	+ 8
D>1.019	22	22	0	13	17	+ 4	12	20	+ 8
Total No. of counts per 30 min.	30,243	35,074		124,000	113,000		14,475	13,213	

the chylomicron fraction was again separated by ultracentrifugation and analysed a second time for triglyceride and cholesterol.

A relative decrease in triglyceride and increase in cholesterol was noted after incubation with serum, this change being more marked in the experiments with CF serum. An absolute increase in cholesterol was also noted in three chylomicron samples incubated with normal serum, but in all cases incubated with clearing factor serum not only was there a marked absolute decrease in triglyceride but also a decrease in cholesterol - except in rabbit 157 (Table 20).

(iv) Electron microscopy

Electron micrographs of the different fractions from rabbit 171 are shown in Fig. 30. On the left are shown the fractions after incubation with normal serum (control) and on the right are fractions after incubation with CF serum. The chylomicrons after incubation with CF serum appear agglutinated and tend to coalesce with an osmium staining irregularity. There is an apparent increase in the number of VLDL and D>1.019 particles in the CF samples with some particles looking L shaped.

TABLE 20

The triglyceride content (TG) expressed in mg/100 ml divided by the cholesterol content (C) in mg/100 ml in the chylomicron fraction of thoracic duct lymph, originally and after being incubated with normal serum and CF serum.

Rabbit No.	Time & temp. incubation	Chylomicrons $\frac{(TG)}{C}$		Chylomicrons after incubation $\bar{c}$ control serum $\frac{(TG)}{C}$		Chylomicron after incubation $\bar{c}$ CF serum $\frac{(TG)}{C}$	
119	2 hr - 20°C	$\frac{1500}{57}$	(27)	$\frac{881}{48}$	(18)	$\frac{260}{35}$	(7)
152	2 hr - 20°C	$\frac{1700}{65}$	(26)	$\frac{1500}{69}$	(22)	$\frac{480}{50}$	(9.7)
153	24 hr - 37°C	$\frac{716}{26}$	(27)	$\frac{600}{68}$	(8.9)	$\frac{50}{16}$	(3.1)
157	24 hr - 37°C	$\frac{1562}{72}$	(22)	$\frac{1248}{132}$	(9.5)	$\frac{676}{127}$	(5.3)

Figure 30

Electron micrographs of the lipoprotein fractions following the incubation of thoracic duct lymph chylomicrons with normal serum (N.S.) and clearing factor serum (C.F.).

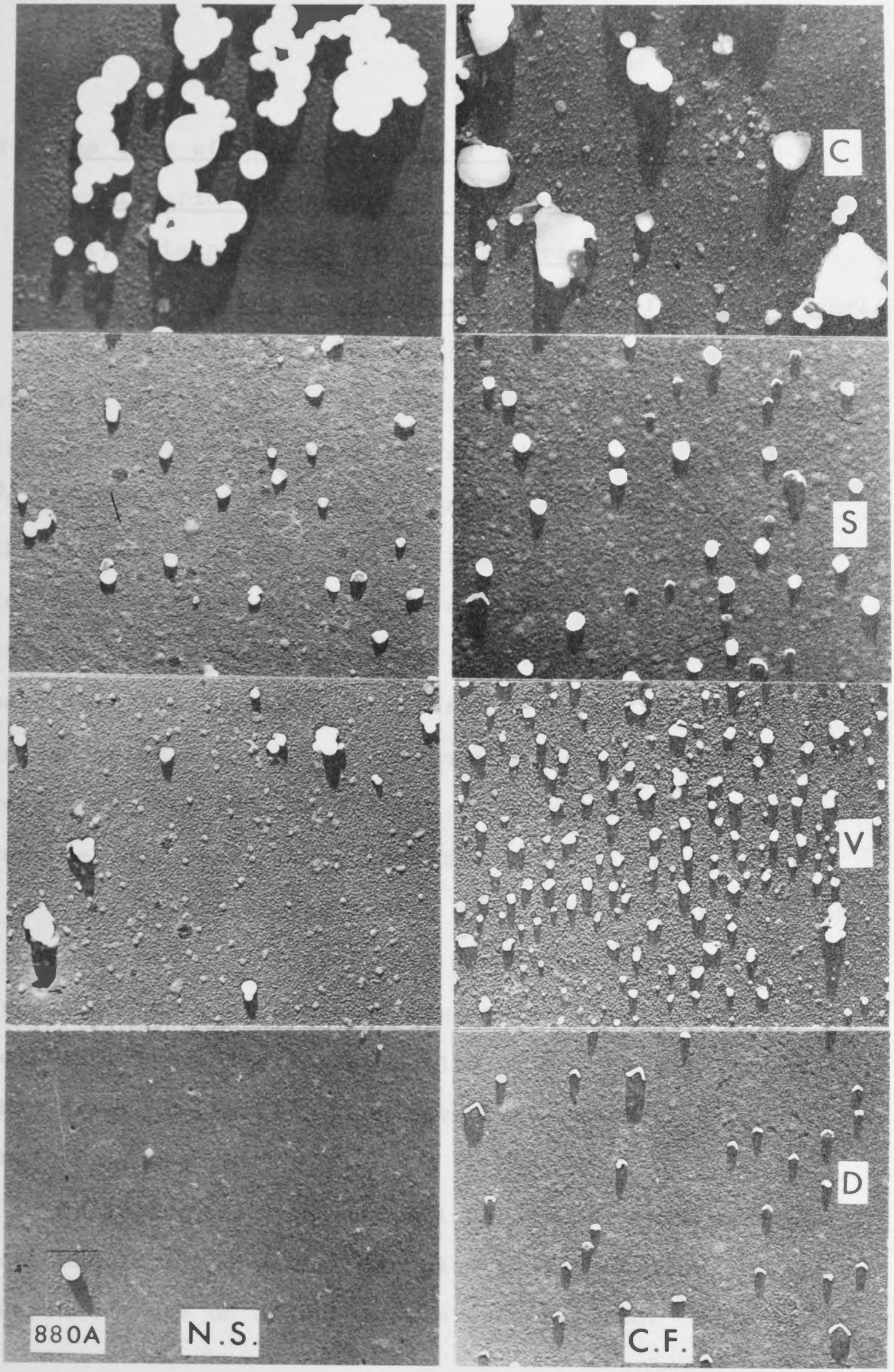
The respective fractions are

C = chylomicrons

S = subnatant I; or saline through which chylomicrons have been centrifuged

V = VLDL

D = D>1.019 lipoproteins


C
S
V
D
880A
N.S.
C.F.

2. The rate of disappearance of the cholesterol and triglyceride component of lipoproteins from the serum

(i) Disappearance of radioactivity from serum following intravenous injection of labelled chyle

The disappearance of radioactivity from the serum of rabbits following a single intravenous injection of thoracic duct lymph lipoproteins labelled *in vivo* with ^{3}H-palmitic acid is shown in Table 21. Also shown is the lipid content and radioactivity of the donor lymph, together with the weight and estimated blood volume of the recipient (Bocci and Viti, 1966) which were used to calculate the approximate initial level of the radioactivity in the serum. The amount of radioactivity actually recovered from the recipient's serum has been expressed as a percentage of the calculated initial radioactivity.

Fig. 31 shows graphically the difference in rates of disappearance of ^{3}H-label from the serum. It can be seen that the radioactivity disappears more quickly following the injection of chylomicrons and whole lymph than after the smaller VLDL have been introduced into the serum.

Following the intravenous injection of chylomicrons and VLDL labelled with ^{14}C-cholesterol a similar, though decreased rate of disappearance was observed. However,

TABLE 21

The disappearance of intravenously injected thoracic duct lymph lipoproteins labelled with ^{3}H-palmitic acid

	Whole lymph		Chylomicrons		VLDL	
Donor Rabbit	522	523	523	523	523	523
lipid content mg/ml	20	7	5	5	2	2
radioactivity counts/ml/min	267,575	224,500	203,000	203,000	40,000	40,000
I.V.I. Injection						
volume ml	2	6	2	1	2.5	3
lipid content mg	40	42	10	5	5	6
counts/minute	535,150	1,347,000	406,000	203,000	100,000	120,000
Recipient rabbit	A	B	C_2	D_1	C_1	D_2
weight kg	1.5	1.5	1.5	1.5	1.5	1.5
estimated plasma vol. ml	60	60	60	60	60	60
initial radio-activity c/ml/min	8,919	22,450	6,767	3,383	1,667	2,000
% recovery of isotope from serum at various times after injection						
1 minute	90	98	57	50	93	75

(continued p.144)

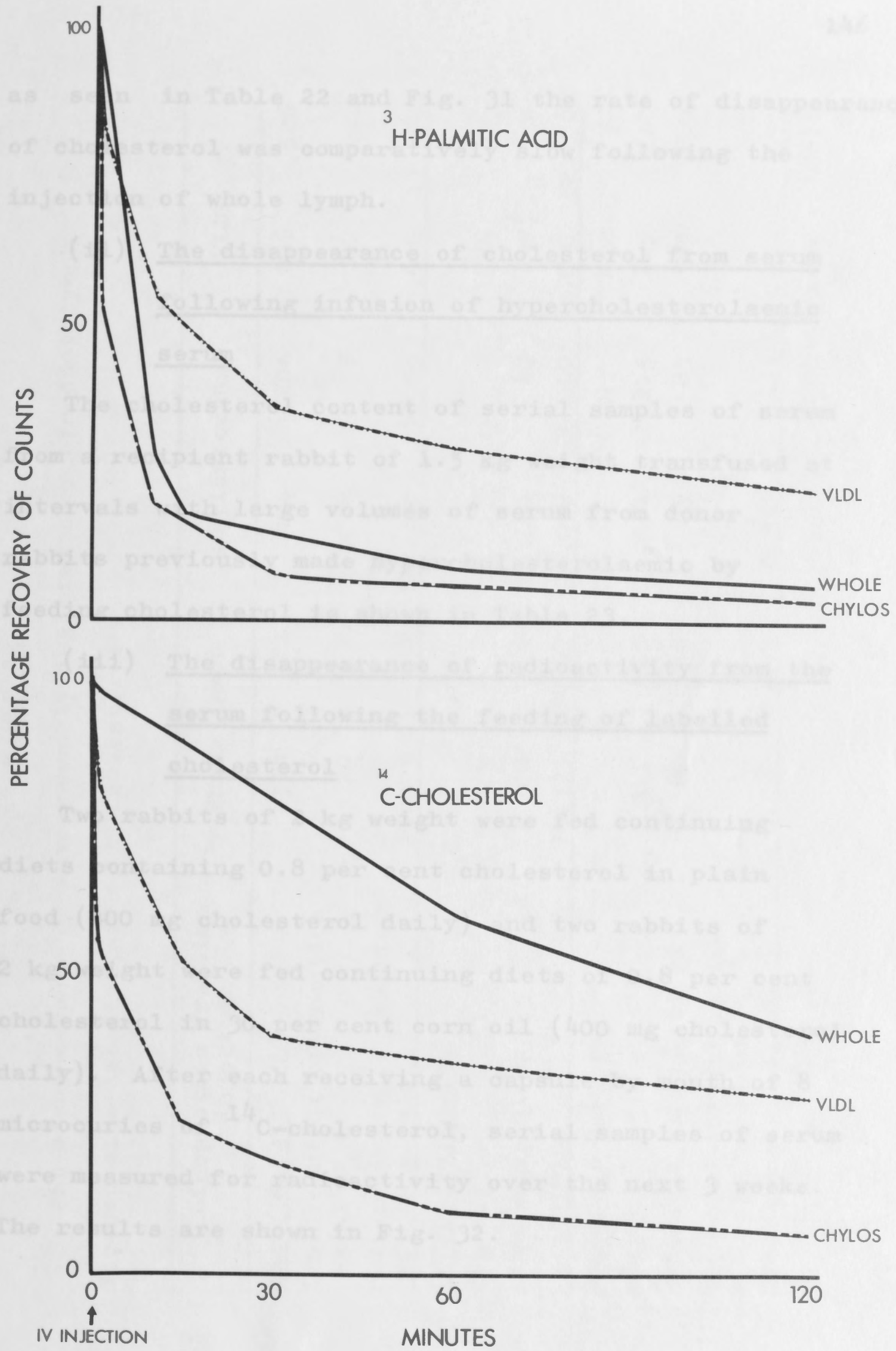
TABLE 21 (continued from p.143)

	Whole lymph		Chylomicrons		VLDL	
10 minutes	33	24	23	17	58	51
15 "	21	16	-	-	-	-
30 "	-	-	6	10	45	28
60 "	12	6	4	7	42	16
120 "	8	4	2	5	30	14
1 day	0	0	0	0	0	0

Figure 31

The disappearance of radioactivity from the blood stream of recipient rabbits following the intravenous injection of <u>in vivo</u> labelled whole lymph, chylomicrons, and VLDL from the thoracic duct lymph of donor rabbits fed ^{3}H-palmitic acid or ^{14}C-cholesterol.

The data for these graphs was taken from Tables 21 and 22.


100
50
0
PERCENTAGE RECOVERY OF COUNTS
3
H-PALMITIC ACID
VLDL
WHOLE
CHYLOS
100
50
0
14
C-CHOLESTEROL
WHOLE
VLDL
CHYLOS
0
30
60
120
IV INJECTION
MINUTES

as seen in Table 22 and Fig. 31 the rate of disappearance of cholesterol was comparatively slow following the injection of whole lymph.

(ii) The disappearance of cholesterol from serum following infusion of hypercholesterolaemic serum

The cholesterol content of serial samples of serum from a recipient rabbit of 1.5 kg weight transfused at intervals with large volumes of serum from donor rabbits previously made hypercholesterolaemic by feeding cholesterol is shown in Table 23.

(iii) The disappearance of radioactivity from the serum following the feeding of labelled cholesterol

Two rabbits of 2 kg weight were fed continuing diets containing 0.8 per cent cholesterol in plain food (400 mg cholesterol daily) and two rabbits of 2 kg weight were fed continuing diets of 0.8 per cent cholesterol in 30 per cent corn oil (400 mg cholesterol daily). After each receiving a capsule by mouth of 8 microcuries of ^{14}C-cholesterol, serial samples of serum were measured for radioactivity over the next 3 weeks. The results are shown in Fig. 32.

TABLE 22

The disappearance of intravenously injected thoracic duct lymph lipoproteins labelled with ^{14}C-cholesterol

Lipoprotein fraction injected	Whole lymph		Chylomicrons		VLDL	
Donor Rabbit	518	521	406	406	406	526
lipid content of fraction mg/ml	20	6	8	8	2	2
radioactivity of fraction counts/ml/min	14,875	17,655	24,000	24,000	3,000	17,390
I.V.I. Injection						
volume ml	5	5	8	5	15	3
lipid content mg.	100	30	64	40	30	6
counts/min	74,375	88,275	192,000	120,000	45,000	52,170
Recipient Rabbit	E	F	G	H_2	H_1	I
weight kg	1.5	1.5	2.4	2.5	2.5	2.2
estimated plasma vol ml	60	60	85	85	85	80
Estimated initial radio-activity of serum c/ml/min	1,239	1,471	2,259	1,412	530	652

(continued p.148)

TABLE 22 (continued from p.147)

Lipoprotein fraction injected	Whole lymph		Chylomicrons		VLDL	
% recovery of isotope from serum at various times after injection						
1 minute	88	107	47	67	84	81
15 minutes	80	100	22	30	53	53
30 "	76	83	12	25	38	42
60 "	65	58	7	14	28	44
120 "	43	42	5	9	24	37
1 day	41	48	48	-	-	-
1 week	37	36	-	-	-	-

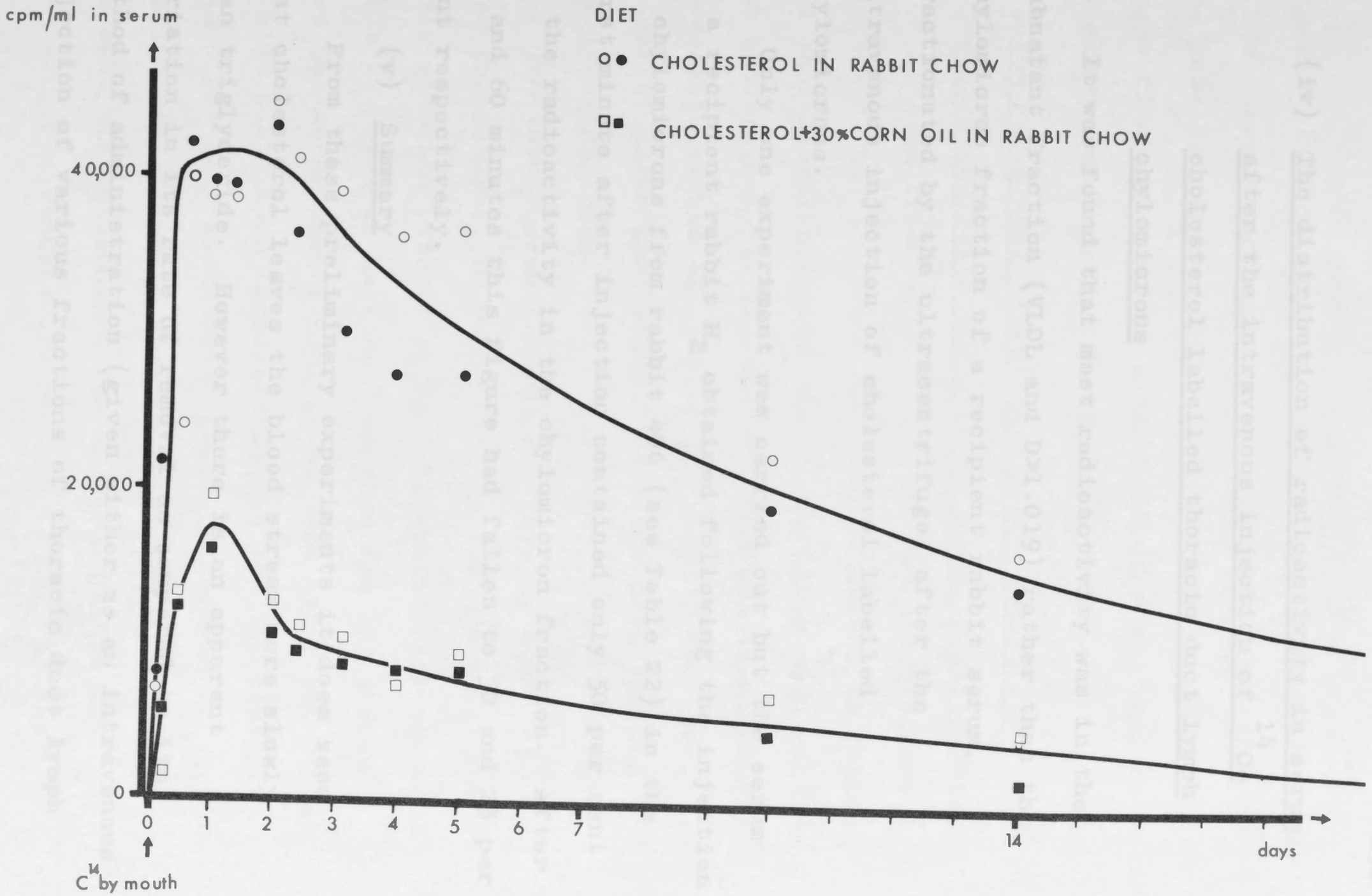
TABLE 23

The clearance of cholesterol from the serum of a recipient rabbit following infusion of serum from a hypercholesterolaemic donor

	Time after infusion	Serum Cholesterol mg/100 ml
Recipient serum before infusion	-	94
First infusion of 20 ml of donor serum	0	1,135
Recipient serum after first infusion	0.5 hr	325
	5	291
	24	201
	48	180
Second infusion of 30ml of donor serum	48	1,500
Recipient serum after second infusion	0.5 hr	559
	6	507
	30	375
	48	327
	5 days	136

Figure 32

The level of radioactivity in the serum of rabbits fed either cholesterol-plain food or cholesterol+30% corn oil following the ingestion of identical amounts of ^{14}C-cholesterol.


cpm/ml in serum
40,000
20,000
0
DIET
CHOLESTEROL IN RABBIT CHOW
CHOLESTEROL+30%CORN OIL IN RABBIT CHOW
0
1
2
3
4
5
6
7
14
days
C14 by mouth

(iv) The distribution of radioactivity in serum after the intravenous injection of ^{14}C-cholesterol labelled thoracic duct lymph chylomicrons

It was found that most radioactivity was in the subnatant fraction (VLDL and D>1.019) rather than the chylomicron fraction of a recipient rabbit serum, fractionated by the ultracentrifuge, after the intravenous injection of cholesterol labelled chylomicrons.

Only one experiment was carried out but the serum of a recipient rabbit H_2 obtained following the injection of chylomicrons from rabbit 406 (see Table 22) in the first minute after injection contained only 50 per cent of the radioactivity in the chylomicron fraction. After 15 and 60 minutes this figure had fallen to 30 and 25 per cent respectively.

(v) Summary

From these preliminary experiments it does seem that cholesterol leaves the blood stream more slowly than triglyceride. However there is an apparent variation in its rate of removal as compared to its method of administration (given either as an intravenous injection of various fractions of thoracic duct lymph

lipoproteins, or serum lipoproteins, or ingested orally) - and these experiments do not exclude re-circulation of the labelled cholesterol in another type of lipoprotein. These findings together with those of other workers will be discussed in detail in the next chaper.

3. The serum cholesterol levels of rabbits fed cholesterol with and without added triglyceride.

As seen from Table 15 preliminary observations suggested that the serum cholesterol levels in rabbits fed cholesterol-high fat diets were lower than rabbits fed cholesterol-low fat diets. This was examined more closely in groups of rabbits fed exactly the same amount of added cholesterol daily but with varying triglyceride loads.

The total serum cholesterol levels, measured as free cholesterol, of young rabbits fed 400 mg cholesterol daily with and without added triglyceride are shown in Table 24. It can be seen that when fed lipid free food the serum cholesterol level does not rise as high as with plain food (cholesterol and low fat diet) or with 5 per cent added triglyceride. However as the triglyceride added is further increased to 15 and 30 per cent the subsequent rise of serum cholesterol is smaller. The results are plotted in Fig. 33 for those

TABLE 24

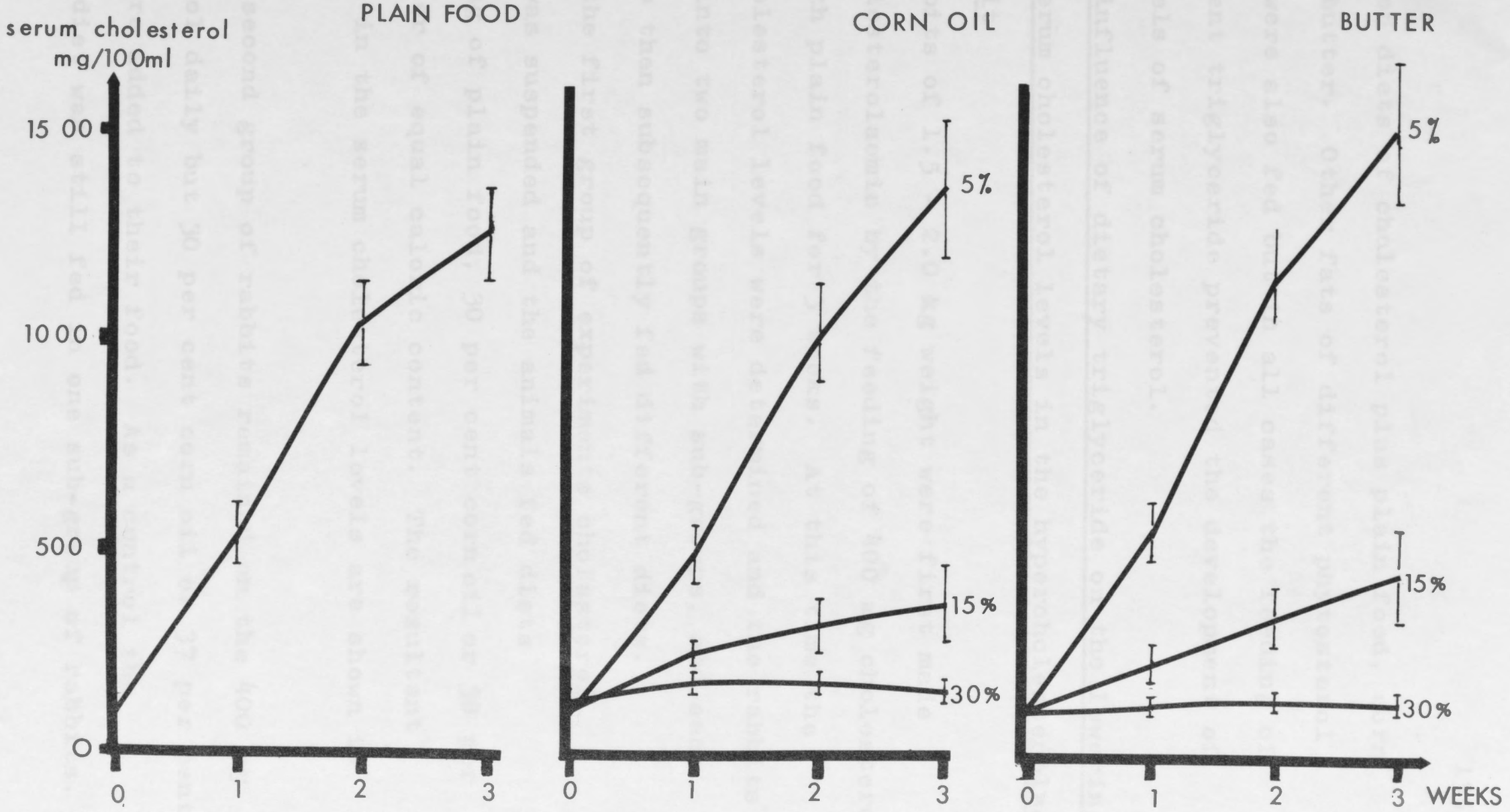
The serum cholesterol levels of groups of rabbits (number in group in brackets) fed identical amounts of cholesterol but with varying triglyceride loads.

DIET 400 mg cholesterol daily plus	Weight of rabbits kg		Serum cholesterol mg/100 ml			
			Weeks on diet			
	week 0	week 3	0	1	2	3
Plain food (9)	2.01±0.05	2.12±0.06	95±27	372±40	620± 70	710± 90
Plain food (9)	1.65±0.07	1.86±0.06	122±24	556±67	1043±116	1248±130
5% corn oil (9)	1.64±0.06	1.86±0.09	136±17	487±57	1031±118	1371±164
5% butter (9)	1.65±0.08	1.91±0.06	126±18	559±72	1152±117	1535±161
15% corn oil (9)	1.73±0.09	1.89±0.07	141±21	225±46	331± 69	357± 90
15% butter (6)	1.75±0.06	-	109±17	247±59	352± 68	448±116
30% corn oil (9)	1.93±0.10	2.13±0.09	167±25	181±27	182± 26	170± 30
30% butter (6)	1.81±0.10	2.00±0.08	127±25	131±20	155± 27	150± 29
30% soya bean oil (3)	1.93±0.07	-	130±17	154±10	131± 14	138± 23
30% Frymasta (4)	1.68±0.06	-	144±13	-	-	203± 21
lipid free food (3) (ether extracted)	1.63±0.07	-	133±25	191±45	370± 60	240± 43

Figure 33

The serum cholesterol levels, recorded at weekly intervals, of groups of rabbits fed 400 mg cholesterol daily with varying triglyceride loads. The data is obtained from Table 24, and each bar represents 2 standard errors of the means.

DAILY CHOLESTEROL 400mg:



animals on diets of cholesterol plus plain food, corn oil and butter. Other fats of different phytosterol content were also fed but in all cases the feeding of 30 per cent triglyceride prevented the development of high levels of serum cholesterol.

4. The influence of dietary triglyceride on the lowering of serum cholesterol levels in the hypercholesterolaemic rabbit

Rabbits of 1.5 - 2.0 kg weight were first made hypercholesterolaemic by the feeding of 400 mg cholesterol daily with plain food for 3 weeks. At this time the serum cholesterol levels were determined and the rabbits divided into two main groups with sub-groups, and each sub-group then subsequently fed different diets.

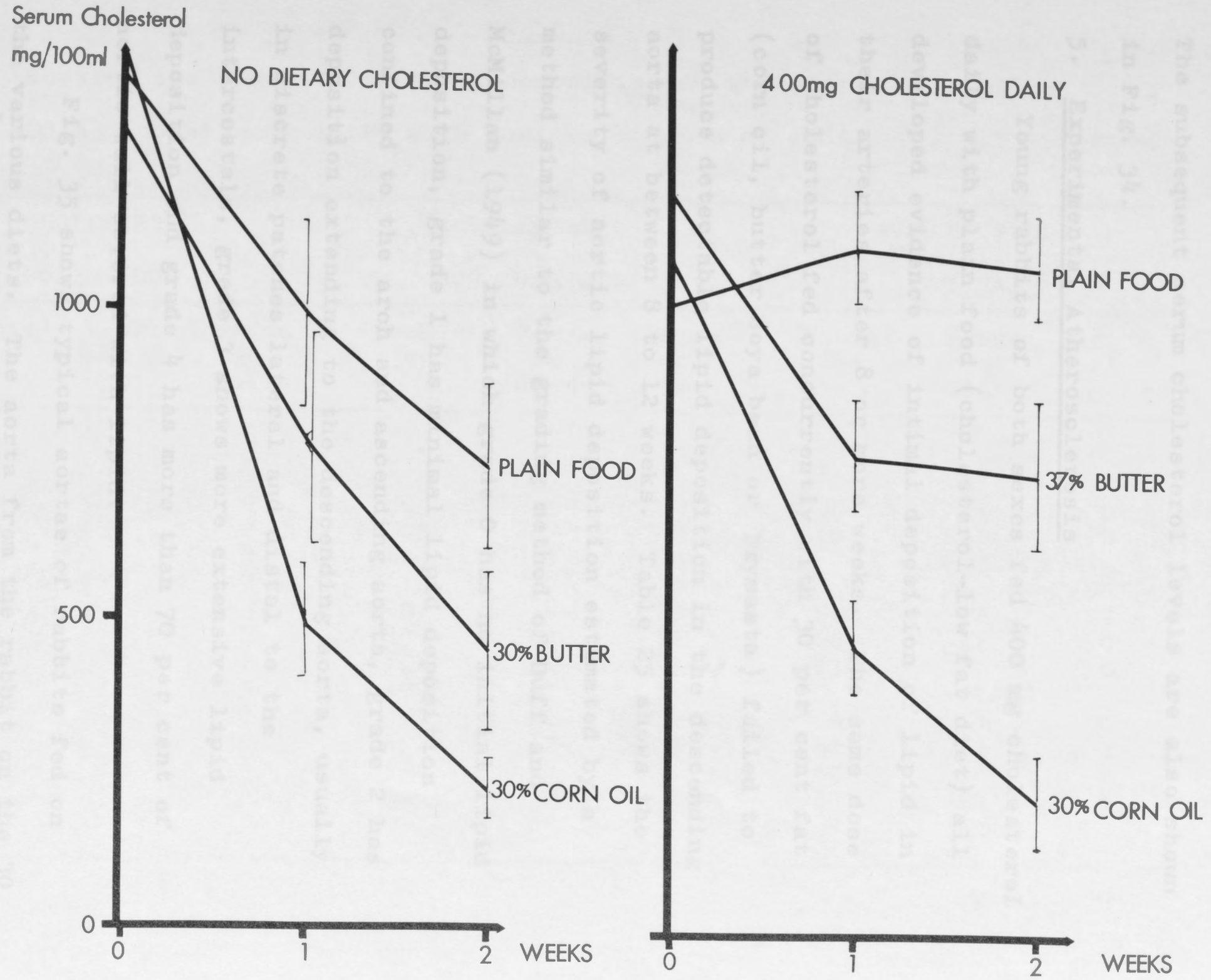
In the first group of experiments cholesterol feeding was suspended and the animals fed diets consisting of plain food, 30 per cent corn oil or 30 per cent butter of equal caloric content. The resultant decreases in the serum cholesterol levels are shown in Fig. 34.

The second group of rabbits remained on the 400 mg cholesterol daily but 30 per cent corn oil or 37 per cent butter were added to their food. As a control the previous diet was still fed in one sub-group of rabbits.

Figure 34

The serum cholesterol levels of groups of rabbits (6 in each group) previously made hypercholesterolaemic by feeding cholesterol in plain food for 3 weeks.

The serum levels are recorded at weekly intervals and in the first graph represent the effect of varying triglyceride loads when no added cholesterol is fed. The second graph represents the effect of varying triglyceride loads when cholesterol is continued in the diet.


Serum Cholesterol
mg/100ml
NO DIETARY CHOLESTEROL
1000
500
0
PLAIN FOOD
30% BUTTER
30% CORN OIL
0
1
2
WEEKS
400mg CHOLESTEROL DAILY
PLAIN FOOD
37% BUTTER
30% CORN OIL
0
1
2
WEEKS

The subsequent serum cholesterol levels are also shown in Fig. 34.

5. Experimental Atherosclerosis

Young rabbits of both sexes fed 400 mg cholesterol daily with plain food (cholesterol-low fat diet) all developed evidence of intimal deposition of lipid in their arteries after 8 or more weeks. The same dose of cholesterol fed concurrently with 30 per cent fat (corn oil, butter soya bean or Frymasta) failed to produce detectable lipid deposition in the descending aorta at between 8 to 12 weeks. Table 25 shows the severity of aortic lipid deposition estimated by a method similar to the grading method of Duff and McMillan (1949) in which grade 0 has no initial lipid deposition, grade 1 has minimal lipid deposition confined to the arch and ascending aorta, grade 2 has deposition extending to the descending aorta, usually in discrete patches lateral and distal to the intercostals, grade 3 shows more extensive lipid deposition and grade 4 has more than 70 per cent of aortic wall covered with lipid.

Fig. 35 shows typical aortae of rabbits fed on the various diets. The aorta from the rabbit on the 30 per cent corn oil cholesterol diet for 12 weeks is

TABLE 25

Showing the severity of lipid deposition in the aortae of rabbits fed 400 mg cholesterol daily with various amounts of additional triglyceride

CHOLESTEROL + PLAIN-FOOD			CHOLESTEROL + 30% CORN OIL			CHOLESTEROL + 30% FAT		
Rabbit No.	Weeks on diet	Grade of aorta	Rabbit No.	Weeks on diet	Grade of aorta	Rabbit No.	Weeks on diet	Grade of aorta
513	4	2	515	4	1		SOYA BEAN OIL	
514	4	1	516	4	1	600	12	0
410	5	0	503	5	0	601	12	0
14	8	2	505	5	0	602	12	0
502	8	2	415	7	0		FRYMASTA	
412	8	2	416	7	0			
402	8	3	I.O.C.	8	0	608	12	1
401	8	2	177	12	1	609	12	0
501	9	2	178	12	1	610	12	0
607	12	3	179	12	0		BUTTER	
606	12	2	590	12	0	606	8	0
605	12	2	591	12	0	603	12	1
180	26	4	592	12	0	604	12	1
			OG	52	3	605	12	0
						OW	52	4

Figure 35

The descending thoracic aortae from rabbits fed

A) cholesterol and 30% corn oil for 12 weeks

B) cholesterol and plain food for 12 weeks

C) cholesterol and plain food for 6 months.

A

B

C

classified as grade 0 while that from the cholesterol plain food diet for 12 weeks is grade 2 showing lipid deposition mainly distal to the intercostal branches. That from the cholesterol plain food diet for 6 months is of grade 4 classification and shows marked lipid deposition with a noticeable exception proximal to the intercostal branches. (Fig. 36)

The coronary arteries from rabbits fed cholesterol and plain food for 8 or more weeks all showed evidence of intimal lipid deposition in some of their branches. The branch most commonly affected involved that running near the papillary muscles of the left ventricle. No coronary arteries from the rabbits fed cholesterol and 30 per cent triglyceride diets, however, were seen to be involved in the first 12 weeks. Fig. 37 shows a typical coronary vessel from a rabbit fed cholesterol and plain food. Positive Schultz staining was also obtained.

The livers of the rabbits were also examined histologically. Fig. 38 represents typical livers from cholesterol-low fat and cholesterol-high fat rabbits and will be discussed later.

6. Summary

The fate of lipoproteins from thoracic duct lymph, and more especially their cholesterol content, once

Figure 36

The descending thoracic aorta at an orifice of an intercostal branch from rabbits

A) fed cholesterol and plain food for 12 weeks

B) fed cholesterol and plain food for 6 months

This illustrates the lipid deposition distal to the orifice and a sparing of the intima proximal to the orifice.

A

B

Figure 37

A typical lipid plaque within the intima of a coronary artery from a rabbit fed cholesterol-plain food for 12 weeks.

The microscopic section was stained with Fettrot.

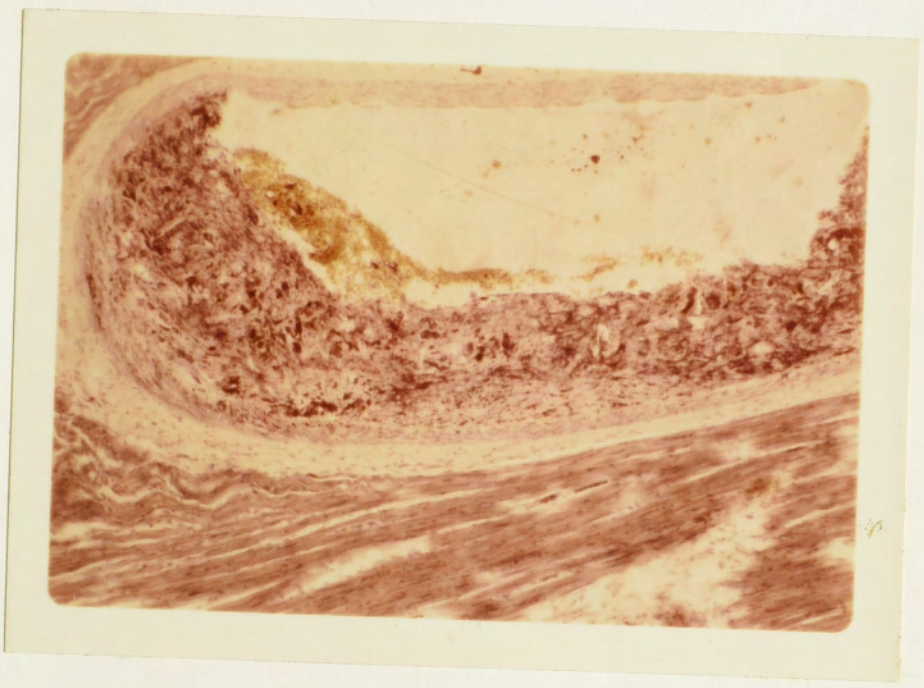
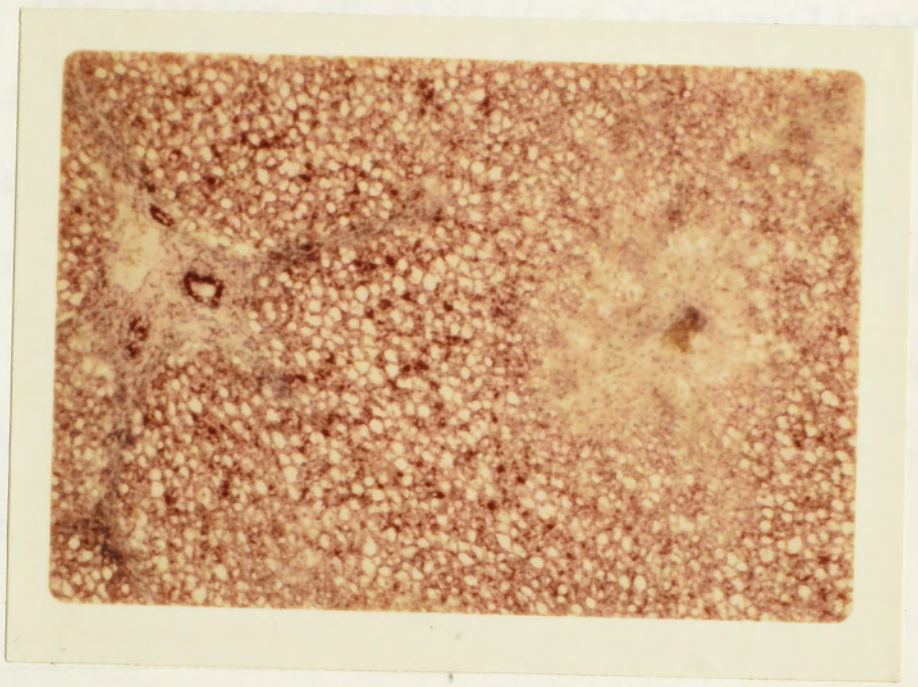


Figure 38

The typical histological appearance of the liver from rabbits fed.

A) cholesterol + 30% corn oil for 12 weeks
(frozen section and Fettrot)

B) cholesterol + plain food for 12 weeks
(frozen section, Fettrot and polarized light)



A

B

they enter the blood stream, has been studied from the aspects of the action of clearing factor lipase, their rate of removal as regards their size, the continuing or re-circulation of their cholesterol moiety in the production of hypercholesterolaemia, and finally their possible role in the production of experimental atherosclerosis. These findings and those of other workers will be discussed in the next chapter.

DISCUSSION

The individual results will be examined for their compatibility with present knowledge and where necessary further hypotheses and experiments will be suggested to clarify the results. A more general discussion of the pathogenesis of atherosclerosis as related to the findings of this thesis will be made, in order to indicate the possible direction of proposed future work.

A. SIZE AND COMPOSITION OF LIPOPROTEINS IN CHYLE

1. The diameter of lipoproteins in the thoracic duct lymph

Early observations of the size of chylomicrons depended on the use of the light microscope. Gage and Fish (1924) found chylomicrons measured about 1μ in diameter and Elkes et al (1939) judged the size of particles in normal human blood to be 1/3-1μ in diameter for "bright" particles, ranging down to 35 mμ for "dull" particles.

Pinter and Zilversmit (1962) measured the size of chylomicrons from differential flotation rates in a sucrose gradient under ultracentrifugation. The median diameters of thoracic duct lymph chylomicrons from dogs fed cream were calculated to lie within the range of

0.18 to 0.29μ It should be pointed out that this median diameter was the diameter of particles at the point of median volume or mass, since triglyceride measurement was used as a marker.

The diameters of chylomicrons can also be measured with the electron microscope. Embedded and sectioned chylomicrons obtained after washing from the chyle of rats were found by Kay and Robinson (1962) to have a wide range of cross-sections varying from 100 to 3,500 mμ. Schoefl (1968) using a similar technique, but without preliminary washing, found the cross sections were smaller, ranging from 25 to 600 mμ, with an occasional chylomicron up to 1,000 mμ. Although the method of sectioning gives information about the structure of chylomicrons, its use in their measurement has the disadvantage that spherical particles are not always sectioned through the centre.

The technique of osmium fixation and metal shadowing has been used in this thesis. This method for the electron microscopic measurement of lipoproteins from human serum and rat chyle was used by Hayes and Hewitt (1957), Garlick et al (1965); Bierman et al (1966) Fraser et al (1968), and Jones and Price (1968). It has the advantage of showing not only the diameter of

lipoproteins but also gives an indication as to the shape of the particles.

(i) Corn oil diet

The fatty acid content of chylomicron triglyceride in thoracic duct lymph, despite some dilution from endogenous lipid, reflects to a large extent the fatty acid pattern of ingested fat (Karmen et al, 1963). Following corn oil ingestion chylomicrons can be fixed with osmium, probably due to the unsaturation of the triglyceride content (Riermersma, 1968; Schoefl, 1968).

The diameter distributions of the chylomicron and VLDL were used as a check on the method of separation by ultracentrifugation. Since many lipoproteins from both these fractions have densities of less than 1.006 (the density of protein free serum), they are separated by their relative migratory habits due to their size and shape, rather than from their density differences. For example if a species of particles having the same density but different sizes is centrifuged in a more dense medium the larger particles will migrate upwards or float faster than the smaller particles (Pinter and Zilversmit, 1962; Dole and Hamlin, 1962).

Dole and Hamlin (1962) stated that the lower limit of particle size in a flotation fraction depended on the

geometry of the centrifuge as well as the amount of centrifugation. For example in an angle centrifuge a flotation of 1 cm usually suffices to bring most of the particles from plasma into an overlying layer of saline. Under these conditions a centrifugation of 10^6 G. min yields a mixture of particles of minimum diameter of about 75 mμ ($S_{f=400}$). The particle diameters, relative density of the medium through which they are being centrifuged, force and time of centrifugation (G. min) are the most important factors in determining the migration of particles. From the nomogram based on Stoke's law in Dole and Hamlin (1962) designed to solve these variables, the minimal diameter of the chylomicrons separated by the methods used in this thesis was calculated to be about 75 mμ.

The lower limit of chylomicron diameter, as determined by electron microscopy, was about 720 Å which compared with the theoretical diameter of 750 Å for particles of S_{f400} (Fig. 9). The VLDL of $S_{f12-400}$ were mostly smaller than 720 Å in diameter with an average of about 500 Å. The D>1.019 lipoproteins were still smaller, few being larger 400 Å in diameter. It can be seen, however, that there is overlap of diameters from each fraction.

Lindgren and Nichols (1960) tabulated the diameters of lipoproteins in human plasma as varying from 800-10,000 Å for particles of $S_{f>400}$, the lipoproteins of S_{f6-8} as being rather asymetrical but with a longest diameter of about 360 Å, and high density lipoproteins as from 100 to 150 Å in diameter. The lipoproteins of rabbit serum ranged from 400-1,400 Å in the D<1.019 fraction (this fraction included the chylomicrons) 200-700 Å in the fraction of density 1.019-1.063 and 100-300 Å in fraction of density > 1.063 g/ml. (Courtice and Garlick, 1962). Further discussion of the sizes of lipoproteins in the serum will occur later in the thesis.

(ii) Cholesterol diet

Similar diameter distributions of the chylomicrons of thoracic duct lymph following cholesterol feeding further confirmed the correlation of electron microscopic estimation of size with theoretical size following ultracentrifugation. (Fig. 10 & 11)

(iii) Butter diet

Present indications are that chylomicrons in the chyle of butter fed rabbits are of the same order of size as following corn oil ingestion (Fig. 7). Indirect measurements of size by comparing the triglyceride/phospholipid ratio tend to confirm this preliminary finding

(Table 7). The importance of determining the size of 'butter' chylomicrons is that at present there is much controversy over their size, which may have some bearing on their ultimate metabolism. It is hoped that freeze etching technique when fully developed will solve this problem and also will be a check on the validity of osmium techniques for the sizing of corn oil chylomicrons (Riermersma 1968). The subject will be further discussed later in the chapter.

2. <u>The effect of dietary fat load on chylomicron and VLDL size in thoracic duct lymph</u>

Gage and Fish (1924) with the light microscope noted that after a fatty meal and especially at the peak of fat absorption not only did the number of chylomicrons increase, but there also appeared to be an increase in their size. Borgström and Laurell (1953) and Simmonds (1955b) came to similar conclusions after comparing the optical density and triglyceride/phospholipid ratios of serial samples of thoracic duct lymph following fat absorption in rats. Casley-Smith (1962) while studying electron micrographs of sections of the intestinal mucosa of rats during the absorption of corn and olive oil, also noted an increase in the relative number of larger lipid particles in the mucosa and lacteals.

An increase in the diameters and more particularly in the volume of chylomicrons in relationship to their diameters was shown in this thesis from the thoracic duct lymph of rabbits and rats fed large doses of corn oil (Fig. 12, 13, 14 and 17). No change in the diameters of the VLDL fraction was noted since the upper limit of VLDL size was limited by the method of centrifugation. (Fig. 15).

These findings firstly may help in further studies to resolve the controversy as to whether chylomicrons following ingestion of saturated fat are larger than those after unsaturated fat, secondly they enable further comparisons between the lipid composition and morphology of chylomicrons, and thirdly may have importance in that the size of lipid particles entering the blood stream may have some influence on rate of removal or the ultimate fate of the component lipids (French and Morris, 1957; Quarfordt and Goodman, 1966, 1967).

(i) Size of 'butter' compared to 'corn oil' chylomicrons

The controversy as to whether 'saturated' chylomicrons are larger than 'unsaturated' chylomicrons may have bearing on the findings of Nestel and Scow (1964) who found that chylomicrons from the thoracic duct lymph of donor dogs and rats, following the ingestion of butter fat,

left the circulation of recipient dogs and rats more quickly than chylomicrons from corn oil fed donors. It was suggested that the main site of this differential removal was the liver and that the physical state of the chylomicrons might play a role in this difference.

Jones et al 1962 using electron microscopic techniques reported large chylomicrons of some 30 microns in diameter after the ingestion of butter in rats, but in 1963 modified their findings by suggesting that poor fixation due to the saturation of the triglyceride component of chylomicrons might have resulted in artefacts. Courel and Clément (1964) with the light microscope found the size of chylomicrons in the chyle of rats was 0.5-1.5μ in diameter after the ingestion of oil or the lipids of cream and 2.4-3μ after the ingestion of fresh cream. Although the authors claimed accuracy with light methods the technical difficulties of distinguishing size at this resolution would appear great.

Using the technique of density gradient centrifugation for measurment of lipid particle size, Zilversmit et al., (1966) found median diameters of chylomicrons of 1770-1950 $\overset{o}{A}$ in the lymph of rats fed cream and 1500-1680 $\overset{o}{A}$ in rats fed corn oil. These were values for the diameter at the median point of chylomicron

mass or volume and not particle number, as ^{14}C-palmitic acid was used as the measure. These values would compare with values in our experiments on lymph from rabbits of 1400 Å on a 5 per cent corn oil diet and 2700 Å on a 30 per cent corn oil diet. In each case half the volume was in chylomicrons with diameters greater than 1400 Å and 2700 Å respectively. Zilversmit *et al.*, did not however, make measurements at varying levels of fat absorption. In our experiments with rat chylomicrons after the ingestion of corn oil, comparable figures were 1320 Å in the early phase of fat absorption and 3600 Å at the peak of absorption.

If freeze etching techniques for the measurement of chylomicrons following the ingestion of butter prove successful, it will be necessary to standardise the load of ingested butter before comparing the sizes of butter and corn oil chylomicrons, since indirect evidence from the triglyceride/phospholipid ratio suggests that the size of 'butter' chylomicrons like 'corn oil' chylomicrons also increases after the ingestion of high doses of butter (Table 7). This will be again discussed in the next section.

3. The composition of chylomicrons and VLDL in relation to their size

Chylomicrons are probably made up of a central core containing predominantly triglyceride with some cholesterol ester covered by a thin surface layer of phospholipid with a little free cholesterol and protein.

This conclusion has been reached firstly from the comparative lipid content of various lipoprotein fractions which indicates that large lipoproteins have a higher $\frac{TG}{PL}$ ratio which is roughly related to the $\frac{V}{SA}$ ratio. (Laurell, 1954; Robinson, 1955; Havel *et al*, 1955; Havel and Fredrickson, 1956; Bragdon, 1951; Dole and Hamlin, 1962; Yokoyama and Zilversmit, 1965).

Secondly Elkes and Frazer (1943) and Robinson (1955) suggested that the stability of serum lipoproteins and of thoracic duct lymph chylomicron emulsion was due to a surface coating of phospholipid and showed that the emulsion could be broken by the addition of phospholipase.

Thirdly electron microscopic studies of sectioned chylomicrons suggest that the bulk of chylomicrons consists of a homogeneous core surrounded by a thin layer or membrane of different electron density (Kay and Robinson 1962, Schoefl 1968, Salpeter and Zilversmit 1968).

Fourthly, studies by Zilversmit (1965) and confirmed by Huang and Kuksis (1967) have shown that after disruption of chylomicrons by freeze thawing techniques the lipid components of the 'core' and 'surface membrane' differed. The membrane weight of the chylomicrons was about 5 per cent of the total weight and contained all of the phospholipid. The membrane was made up of approximately 75 per cent phospholipid, 13 per cent triglyceride, 5-10 per cent free cholesterol and 0.4-4 per cent protein. No cholesterol ester was found in the membrane fraction. The core, however, contained about 90-95 per cent triglyceride, some cholesterol ester, only a little free cholesterol and no phospholipid.

The composition of phospholipid in chylomicrons from thoracic duct lymph has been shown to consist mainly of lecithin (Whyte et al 1963, Zilversmit 1965) whereas in serum there is comparatively less lecithin and more sphingomyelin. Zilversmit (1968a) from studies on the composition of phospholipids and their fatty acids in thoracic duct lymph chylomicrons, concluded the phospholipid was probably derived from within the mucosal cell of the intestine rather than from the surrounding tissue fluids or serum after the chylomicron had escaped from the mucosal cell. The absence of a

double layer on the membrane as seen by electron microscopy also suggested that the surface membrane of chylomicrons was not derived from the outer layer of the mucosal cells (Salpeter and Zilversmit 1968).

The results of the chemical composition found in this thesis are consistent with the findings that chylomicrons from thoracic duct lymph of both rabbits and rats increase in size with increasing absorptive fat loads. Although these particles vary considerably in size, phospholipid if uniformly spread seems to form a monomolecular layer on their surface, each phospholipid molecule occupying the same area, about 65 A^{o2} on the average. This applies also to VLDL. The layer is probably a mixed film consisting mainly of phospholipid but also some cholesterol and protein which would give a more compact film (Zilversmit, 1965; Van Deenen et al., 1962; Demel et al, 1967; Lossow et al, 1967).

(i) 'Butter' chylomicrons compared to 'corn oil' chylomicrons

The area occupied by a phospholipid molecule in a monomolecular layer probably varies with its fatty acid compositon. Anderson and Pethica (1956) showed that in a monomolecular film of an artificial lecithin, at an

air-water interface, each lecithin molecule took up an area of 40 $\overset{o}{A}$ at closest stable packing. Van Deenen *et al*, (1962) and Demel *et al*., (1967) confirmed this, but have shown also that if the fatty acids of lecithin were unsaturated, the lecithin molecule took up a larger area. For example, they found a lecithin molecule with one stearic and one oleic fatty acid took up an area of about 70 $\overset{o}{A}^2$ in a closely packed monomolecular layer. It is possible, therefore, that at a given $\frac{TG}{PL}$ ratio, chylomicrons would be a little smaller if polyunsaturated fatty acids were present in the lecithin with a resultant increase in area taken up by phospholipid molecules. Similarly, theoretical size variations of chylomicrons with mixed films on their surfaces are possible since cholesterol and unsaturated lecithin cover different areas from a cholesterol and saturated lecithin (Demel *et al*., 1967).

Whyte *et al* (1963) showed that 75 per cent of phospholipid in rat chylomicrons was lecithin and that approximately 35 per cent of the total fatty acids in lecithin was derived from the diet. The density of packing of lecithin molecules in a monomolecular layer on the surface of chylomicrons from butter fed animals may, therefore, be different from that on chylomicrons

from corn oil fed animals. However, the difference in size of chylomicrons which could theoretically be caused by the difference in packing area of lecithin molecules would not give rise to such a variation in size as obtained by some observers (Jones *et al.*, 1962). Since in our experiments the $\frac{TG}{PL}$ ratios were similar for corn oil and butter chylomicrons (Table 7), it seems likely that there is no great difference in size. Our results are, therefore, more in agreement with those of Zilversmit *et al.* (1966), but remain to be confirmed by electron microscopy.

(ii) Formation of chylomicrons

In the formation of chylomicrons by the mucosal cells the rate of phospholipid synthesis could be a factor accounting for the variation in size. Palay and Karlin (1959) noted the close relationship of endoplasmic reticulum of the mucosal cell to the formation of chylomicrons and their transport to the sides of the cell before discharge into the extracellular fluid. The present theory of fat absorption suggests that there is a passive diffusion of fatty acids, mono- and di-glycerides into the cell and then an active synthesis of triglyceride, phospholipid and protein by the endoplasmic reticulum or Golgi complex to form

chylomicrons (Senior and Isselbacher, 1962; Sjöstrand, 1963; Hofmann and Borgström, 1964; Cardell et al., 1965; Strauss, 1966; Dobbins, 1966).

Redgrave (1967) in his studies on micellar absorption in the rat intestine suggested that the last step, the transport of chylomicrons from the mucosal cell into the lymph might well be a limiting factor in fat absorption. Zilversmit et al., (1966) postulated that chylomicrons formed in the mucosal cell might at first be sparsely covered with phospholipid. After discharge from the cell, however, they might coalesce to form larger particles, thus decreasing the surface to volume ratio and leading to tighter packing of the phospholipid molecules.

Our findings indicate that in the thoracic duct lymph the area occupied by each phospholipid molecule on the surface of chylomicrons and VLDL is the same irrespective of size of particle. If the rate of phospholipid synthesis in the mucosal cell is a limiting factor, an increase in the size of chylomicrons could be a mechanism for transporting a greater triglyceride load.

B. THE SIZE AND COMPOSITION OF PARTICULATE LIPID IN VARIOUS ARTIFICIAL FAT EMULSIONS COMPARED WITH CHYLOMICRONS

A fat emulsion suitable for intravenous nutrition has been sought for many years (Geyer, 1960). Recently soya bean oil emulsions have been used clinically in the treatment of anuria, burns and other illnesses where the need for parenteral calories without oral feeding is indicated (Lee and Sharpstone, 1967; Hallberg et al, 1967). Synthetic emulsions made from other oils have also been used in man (Watkin, 1957; Mueller and Viteri, 1965). In Australia little clinical use has been made of these emulsions because of fear of toxicity, but recently paediatric surgeons in Sydney have shown interest as they would provide an ideal method of feeding high calories to babies where fluid balance is a particular problem (Owen E.R., 1968 personal communication).

An ideal artificial fat emulsion not only must be stable enough to withstand sterilization, transport and storage (McQuarrie and Andersen, 1965; Håkansson, 1966) but also should be removed from the blood stream for metabolism in a manner similar to chylomicrons (French and Morris, 1957; Carlson and Hallberg, 1963; Hallberg

1965a and b, Håkansson, 1968). It should also be of low toxicity (Wretlind, 1964; Rose et al, 1965; Thompson et al, 1965; Hadfield, 1967; Håkansson 1968).

The shake and transport stability of Intralipid, for example, has been improved by the recent manufacture at a more alkaline pH. The stability has been found to be greater in a pH range between 5.0 - 6.5 and 7.5 - 9.0 than at a pH of about 7. Since all emulsions are systems prepared with the input of energy, so the energy level is higher for emulsions than for separated phases, and therefore in time all emulsions will tend to be broken down. During the ageing of emulsions like Intralipid a very small spontaneous hydrolysis takes place leading to a liberation of free fatty acids causing a drop in pH. Hydrolysis is minimal at a pH of about 6.8 but increases on either side of this pH. The new Intralipid emulsions therefore have been manufactured at a pH above this level, since if emulsions are prepared at a pH of about 8 the hydrolysis rate will decrease with time since the pH will come nearer to the point of minimal hydrolysis. If on the other hand emulsions were prepared at a pH of below 6.0 - 6.5 the hydrolysis rate would increase with time (Håkansson 1968, personal communication).

The kinetics of removal of particulate lipid from the blood stream have been extensively investigated both for chylomicrons and artificial fat emulsions. The disappearance rate of particulate lipid of artifical fat emulsions from the blood stream in man and experimental animals has been studied by Meng and Freeman, 1968; Meng, 1952; Becker et al, 1955; McCandless and Zilversmit, 1958; Carlson and Hallberg, 1963; Forbes 1965; and Hallberg, 1965a.

Carlson and Hallberg (1963) for example found the kinetics for the elimination of both Intralipid 10 per cent and thoracic duct lymph chylomicrons from the blood stream of dogs to be similar; while in man Hallberg (1965a and b) found that Intralipid 10 per cent followed a similar pattern of elimination kinetics as Bierman and Hamlin (1962) and Nestel et al (1962a) found for the elimination of C^{14} labelled triglyceride particles and thoracic duct lymph chylomicrons in man.

Factors which may alter the kinetics of elimination of chylomicrons and lipoproteins from the blood stream may include size (French and Morris 1957, Quarfordt and Goodman, 1966a and b, 1967) triglyceride saturation (Nestel and Scow, 1964) amount of fat injected (French and Morris, 1957) and the nutritional state of the animal

(Morris 1958). Similar factors probably play a part in the rate of elimination of artificial fat particles from the blood stream.

1. The size of particulate lipid in artificial fat emulsions

The size of the lipid particles is probably of importance in their clearance (French and Morris 1958) and in their long term toxicity, such as liver cell damage (Thompson et al, 1965) and acute anaphylactic like reactions (Atik et al, 1965). As with chylomicrons various methods have been used to measure the diameters of particulate lipid in artificial fat emulsions including the use of the sucrose gradient (Pinter and Zilversmit, 1962), the Coulter Counter for large particles (Atik et al, 1965) and more recently Schoefl (1968) has examined sectioned particles by means of the electron microscope.

The diameter distributions of particulate lipid as found in this thesis show two methods of alteration of size at manufacture. Fig. 20 and Table 8 illustrate the effect of altering the triglyceride-phospholipid ratio. Particles from SBO A are larger than from SBO B, SBO C and SBO D suggesting that as more phospholipid is added the particles become smaller due to the presence of more

phospholipid to stabilize their larger surface area. However there does appear to be a lower limit to the size of particles since as further phospholipid is added the particle diameters again increase as in SBO F and SBO G.

Another factor apparently altering the size of particulate lipid is the pH at manufacture. As explained earlier the new Intralipids were manufactured at a more alkaline pH than the old for greater stability in storage. It was noted however that the particles in the new Intralipids appeared smaller than in the old Intralipids (Fig. 21). To determine whether this was just an effect of ageing or due to the different pH of manufacture, fresh batches of experimental soya bean oil emulsion were made and the size distributions determined. Fig. 22, 23 and 24 show that artificial fat emulsions of identical triglyceride-phospholipid composition contain smaller particles if manufactured at an alkaline pH. The explanation for this finding is not known but may be due to electrical charges in different parts of the system during the process of manufacture (Hökansson 1968, personal communication).

The stabilizing surface active agent in artificial lipid particles may be of importance in their elimination from the blood stream and metabolism (Waddell et al, 1953;

French and Morris, 1958). The structure of artificial fat emulsions resembles chylomicrons since from electron microscopic observations they contain a central core probably made up of triglyceride, and a thin membrane presumably containing phospholipid (Schoefl 1968). The area taken up by each phospholipid molecule on the surface of lipid particles of different fat emulsions was calculated on the theoretical assumption that the phospholipid was evenly spread over the surface of all the particles in the emulsion (Table 9). It can be seen that whereas SBO A-C probably contain phospholipid as a monomolecular layer on their surfaces the density of packing increases in SBO D-G suggesting that the molecules are heaped on each other. Schoefl (1968) noted from electron microscopic sections of Intralipid 20 per cent an occasional area of reduplication of the surface layer; while in SBO G a laminated surface layer, presumably containing phospholipid, was apparent (Schoefl, 1968, personal communication).

The size and surface composition of artificial fat emulsion particles not only may affect their disappearance from the blood stream but also the sites of uptake, metabolism and possible toxicity. For example, the distribution of fat in the body after intravenous fat

emulsion was found to be in the reticulo-endothelial system of the liver, spleen and lungs whereas after infusion of chylomicrons it was more in the parenchymal cells of the liver (French and Morris, 1958). Schmidt-Diedrichs and Courtice (1963) also found a difference in the fate of chylomicrons and Lipomul particles from the doubly ligated carotid artery of the rabbit in that the artificial particles produced a foreign body reaction.

2. The sterol content of artificial fat emulsions

Small traces of other components might also affect the toxicity of artificial fat emulsions. Rose et al (1965) analysed the components of various artificial fat emulsions because of their conviction that improvement of intravenous fat nutrition depended to a considerable extent on the exact knowledge of the materials present in intravenous emulsions, including impurities and minor components. The presence of campesterol, stigmasterol, sitosterol and other unidentified sterols was noted in both soya bean and cotton seed oil emulsions by these workers.

The presence of sterols not only from the egg source but also from the soya bean source was also noted in this thesis (Table 10). In nature little phytosterol

is absorbed by animals and man (Fraser and Gardener, 1910; Schoenheimer, 1931; Gould et al, 1953; Borgström, 1967; Grundy et al, 1968) and it is interesting to speculate that some of the delayed toxic effects of intravenously injected artificial fat emulsions might be due to these sterols. For example, one toxic effect after prolonged intravenous therapy is liver damage in which at first pigment deposition is seen in the hepatocytes of the pericanular zone eventually leading to electron microscopic changes of the mitochondria and dilation of the endoplasmic reticulum. The source and composition of the pigment is not yet known, but from staining appears to be lipid (Sasaki et al 1965). Lipomul (in which both the triglyceride and the phospholipid component are derived from vegetable origin, and thus all its sterol content would be phytosterol) causes this reaction more markedly than Intralipid; while an artificial fat emulsion tested by Thompson et al (1965) and made up of synthetic triglyceride and stabilizer, and so without phytosterol, although very toxic did not produce these fat pigment deposits.

C CHOLESTEROL IN LYMPH AND SERUM

The absorption and transport of cholesterol has been studied in some detail because of its association with arterial lipid deposition in the cholesterol fed rabbit.

1. Cholesterol in thoracic duct lymph

It has long been known that all absorbed cholesterol reaches the blood stream from the intestine via thoracic duct lymph (Mueller, 1915; Chaikoff et al 1952). However, not all absorbed cholesterol is derived from the diet but some comes from the bile and also from excretion by the intestinal cells into the gut lumen from which it may be reabsorbed (Sylvén and Borgström, 1968; Reinke and Wilson, 1968).

Early workers believed that cholesterol could neither be synthesised nor destroyed within the body. Gardner and co-workers in a series of papers on the origin and destiny of cholesterol in animal organisms presented to the Royal Society from 1908 to 1913 were of this opinion. However, Bloch and Rittenberg (1941) showed that acetate could be used for the in vivo synthesis of cholesterol. Substantial amounts of cholesterol have been shown to be synthesised in other tissues, notably the liver (Bloch et al, 1946; Gould and Taylor, 1950; Hotta and Chaikoff, 1955) and the intestine (Gould, 1951; Lindsey and Wilson, 1965; Wilson and Reinke, 1968). Nearly all tissues to a lesser extent have been shown to be capable of cholesterol

synthesis (Srere et al, 1950) including the macrophages to be found in atherosclerotic arterial intima (Day, 1967). However, probably only liver and intestinal synthesis contribute to the endogenous cholesterol in the serum and it has been suggested that this also reaches the blood via thoracic duct lymph (Wilson and Reinke, 1968).

Dietary cholesterol has been shown to depress cholesterol synthesis in the rat's liver (Gould et al, 1953; Tomkins et al, 1953; Morris et al, 1957) but not in the rat's intestine (Wilson and Reinke 1968). Dietary cholesterol has not, however, been shown to suppress cholesterol synthesis in man (Lindsey and Wilson, 1965). Dietary cholesterol supresses liver synthesis, but not intestinal synthesis, in the rabbit (Gould, 1951; Popják and Beekmans, 1950).

These different sources of cholesterol in thoracic duct lymph together with the possibility of some cholesterol reaching the lymph from the peripheral blood stream, especially when carried in the smaller lipid particles of D>1.019, (Zilversmit et al, 1967) make the interpretation of cholesterol present in lymph difficult.

Zilversmit et al (1967) in studies of cholesterol transport in thoracic duct lymph of rabbits fed mainly

cholesterol-low fat diets remarked that a higher proportion of cholesterol was carried in the VLDL fraction than in the chylomicron fraction. In the rat fed 100 mg of cholesterol in 3 ml corn oil however, Byers and Friedman (1954) found the chylomicron fraction of thoracic duct lymph contained 96 per cent of the lymph cholesterol, and in the dog fed 30 ml corn oil and 0.5 g cholesterol Hillyard et al (1958) found that chylomicrons ($S_{f10^3-10^5}$) contained ten times as much cholesterol as VLDL ($S_{f20-1000}$). Zilversmit (1968b) in studies on thoracic duct lymph of dogs fed corn oil, with and without cholesterol, showed newly absorbed cholesterol was mainly in the chylomicron fraction as cholesterol ester.

Studies in this thesis of the cholesterol content of thoracic duct lymph in rabbits fed either cholesterol-low fat or cholesterol-high fat diets indicate that the cholesterol entering the rabbits' blood stream from the lymph varies in the size of the particle in which it is carried. For example relatively more cholesterol is transported in the chylomicron fraction than in the VLDL fraction of the chyle of rabbits fed cholesterol-high fat diet, while the reverse is true in rabbits fed cholesterol-low fat diet. (Fig. 26, 27 and 28).

The total cholesterol content of chyle from rabbits fed cholesterol-low fat diet was also found in this thesis to be higher than in rabbits fed cholesterol-high fat diet (Table 11). Measurements of lymph flow were not carried out, but in experiments on the lymph flow of the rat Borgström and Laurell (1953) and Simmonds (1954) noted a twofold increase in thoracic duct lymph flow during fat absorption. If a similar increase in flow occurred in the fat-fed rabbit then the total cholesterol reaching the blood stream in a given time would be approximately equal in the two groups of animals.

Experiments show a similar trend in the distribution between the chylomicron and VLDL fractions of chyle from rats fed cholesterol-low fat and cholesterol plus 30 per cent corn oil (Table 12).

Recently Ockner and Isselbacher (1968) have shown the cholesterol content of chyle from fasting rats to be mainly present in the VLDL fraction. After bile fistula or the intra-duodenal administration of cholestyramine, they found a prompt fall in lymph total cholesterol levels and virtual disappearance of cholesterol in the VLDL and chylomicron fraction. This they felt indicated that both chylomicron and VLDL cholesterol were derived

from the intestinal cells rather than as a filtrate from circulating lipoproteins. Furthermore, they found the fall in lymph cholesterol exceeded the amount diverted from the intestine in bile fistula rats, which they suggested supported the concept that portion of the lymph cholesterol is synthesised in the intestine. They also found that when intraduodenal unsaturated fatty acids were administered as mixed micelles, the cholesterol content of the thoracic duct lymph was redistributed in that the majority of cholesterol was now in chylomicron fraction. If, however, saturated fatty acids were administered the cholesterol was still mainly in the VLDL fraction of thoracic duct lymph.

In experiments in this thesis the distribution of ingested ^{14}C-cholesterol in the lipoprotein fractions of rabbit chyle also indicated that in the cholesterol-high corn oil fed animals relatively more cholesterol was present in chylomicrons than in the cholesterol-low fat fed animal (Table 13). It was apparent however that the proportions of ^{14}C-cholesterol varied from the cholesterol when measured chemically (Table 14) in that in all cases more radioactivity was present in the chylomicron fraction; except in one case where lymph was collected over the second twelve hours after cannulation.

The increased amount of chemically measured cholesterol in the D>1.019 fraction when compared with the radioactivity present supports the idea that the cholesterol in this fraction does not all represent absorbed cholesterol, but comes from some other source such as filtrate from the peripheral blood (Zilversmit et al, 1967; Courtice, 1968; Ockner and Isselbacher, 1968). The increased amount of chemically measured cholesterol as compared to the radioactive cholesterol in the VLDL fraction may also be due to the same explanation. However this appears less likely, since the VLDL of serum appear larger in the electron microscope than the VLDL of chyle (Fig. 10 and 29), and are probably too large to filter through the peripheral capillaries in any quantity (Courtice and Garlick, 1962). Another explanation maybe that some of the cholesterol in the thoracic duct VLDL represents cholesterol synthesised in the intestine or liver (Reinke and Wilson, 1968). A third explanation is that cholesterol in the VLDL fraction might represent a transport mechanism of ingested cholesterol in a later phase of absorption, as suggested by rabbit 6 (2) in Table 13.

The results discussed in this section show that the distribution of total cholesterol in thoracic duct lymph

of rabbits and rats varies when cholesterol is fed with low fat or high fat diets. The source of the cholesterol is un-proven but most of the cholesterol in the chylomicrons and VLDL probably represents cholesterol from the rabbit's intestine. Experiments necessary to investigate this problem further include the measuring of free and esterified cholesterol, and the use of isotopes including such techniques as intraduodenal administration of micellar solutions of cholesterol with and without added fat. A chronic experimental rabbit with a return loop thoracic duct fistula is an ideal yet to be realised, to determine if there is a difference in the fraction of chyle in which cholesterol is absorbed in the early and late phase.

2. <u>The distribution of cholesterol present in serum of rabbits fed cholesterol with varying triglyceride loads</u>

As has been seen cholesterol enters the blood stream via the thoracic duct lymph in triglyceride rich lipoproteins of the chylomicron and VLDL class. When Tables 11 and 15 are compared as in Table 16, it can be seen that the lipoproteins of the plasma are richer in cholesterol and that much of the cholesterol, especially in the hypercholestesteolaemic serum of the rabbits fed

cholesterol and plain food, is in the VLDL fraction. Garlick and Courtice (1962) also noted that the cholesterol was carried especially in the lipoproteins of D<1.019 g/ml and that this fraction accounted in the main for the cholesterol content of serum with high levels of cholesterol.

French et al (1955) noted that the plasma lipoproteins of rabbits fed cholesterol contained a much greater relative composition of cholesterol than did the chyle, and found that although clearing factor lipase cleared the turbidity in chyle it has no effect on the turbidity of plasma. They suggested that the lipaemia of rabbits fed cholesterol could be attributed primarily to the failure in the disposal of the cholesterol-containing residue of the lymph chylomicra rather than a failure in the mechanism for the breakdown of these particles in blood. The lipoproteins of the serum of hypercholesterolaemic rabbits might thus be regarded as "burnt out" thoracic duct lymph lipoproteins.

Fig. 29, the electron micrograph of the three fractions of serum from a hypercholesterolaemic rabbit fed cholesterol-plain food, shows the sizes of lipoproteins. The lipid particles were rather flattened so no accurate attempt to plot a percentage distribution was made, but

it was noted by comparing Fig. 29 with Fig. 10, that the particles of the VLDL fraction in serum appeared bigger than in chyle.

Although the apparent greater size of serum VLDL might be artefactual due to poor fixation, they correspond well with the size distribution of lipoproteins in hypercholesterolaemic rabbits found by Courtice and Garlick (1962). These authors found that lipoproteins of D<1.019 g/ml ranged in diameter from less than 400 $\mathring{A}$ up to over 1200 $\mathring{A}$ and although this fraction included chylomicrons it can be seen from Table 15 that the chylomicron fraction is relatively small in serum.

A possible explanation of the larger size of VLDL in hypercholesterolaemic serum is that the cholesterol content of this fraction is high, averaging about 66 per cent of the total lipid (Table 16) which is approximately the same as that found by Garlick et al (1965). The density of anhydrous cholesterol is 1.06 g/ml and of its monohydrate form with water is 1.04 g/ml (Rose, 1968); so an increase in density due to cholesterol might be sufficient to prevent particles of diameter larger than 750 $\mathring{A}$ behaving like chylomicrons of $S_{f>400}$ in their flotation characteristics.

The serum of hypercholesterolaemic rabbits is well known to be turbid due to light scattering lipoproteins of D<1.019 g/ml (French et al, 1955; Garlick and Courtice, 1962) but unlike the chyle, the turbidity is not removed by ultracentrifugation of the order to remove particles of $S_{f>400}$. The finding that serum VLDL particles are comparatively larger than chyle VLDL may explain the turbidity caused by these particles in hypercholesterolaemic rabbit serum.

The percentage lipid composition of the D>1.019 fractions of chyle and serum can also be compared in Table 16. It can be seen that in this fraction also there is comparatively more cholesterol and less triglyceride in the serum compared to the thoracic duct lymph. This suggests that the D>1.019 lipoproteins of chyle are not all derived as a filtrate from serum. The D>1.019 fraction contains lipoproteins from a wide spectrum including low density of S_{f0-12} and the high density lipoproteins, and it seems possible that some of this spectrum maybe derived from the intestine.

D. SOME ASPECTS OF THE FATE OF LIPOPROTEINS AFTER ENTRY INTO THE BLOOD STREAM

From the last section it can be seen that whereas lipoproteins enter the blood stream from the chyle of rabbits mainly as triglyceride-rich chylomicrons the predominant lipoproteins found in the serum of hypercholesterolaemic rabbits are VLDL or D>1.019 particles with comparatively less triglyceride and more cholesterol. This section deals with attempts to explain this phenomenon and to trace the lipoproteins, and especially their cholesterol component, to their possible fate in the arteries.

1. The action of clearing factor lipase on thoracic duct lymph chylomicrons

The *in vitro* action of clearing factor lipase on chylomicrons was studied in view of the relative decrease in triglyceride content and size of lipoproteins in the serum of hypercholesterolaemic rabbits compared to the lipoproteins entering the blood stream from the thoracic duct, and because of the suggestion by French *et al* (1955) of the role of clearing factor lipase in this change.

Clearing factor lipase or a similar enzyme has been isolated from a number of tissues of animals and it is thought that this enzyme plays a major part in regulating the uptake of triglyceride fatty acids from the blood stream *in vivo* (Robinson 1963). For example it has been shown that when heparin is injected into humans and rabbits there is a transitory reduction of the $S_{f20-40,000}$ lipoproteins of relatively high TG content with a concomitant increase in the S_{12-20} class (Graham *et al*, 1951). Similar changes in lipoprotein pattern were observed *in vitro* after incubation of lipoproteins with plasma obtained from animals previously injected with heparin (Anfinsen *et al* 1952, Boyle *et al* 1952, Lindgren *et al* 1952, Lindgren *et al* 1955). The changes brought about in these experiments are thought to be due to the action of a heparin activated lipase, "clearing factor". This enzyme system has been shown to be capable of clearing lipaemic plasma and of hydrolysing chylomicron triglycerides to glycerol and free fatty acids (French *et al* 1953), which are then bound to albumin to give an optically clear solution of glycerol and fatty acids (Robinson and French 1953).

In view of the transformations that occur in the composition of plasma lipoproteins in the presence of

clearing factor *in vivo*; Lindgren *et al* (1956) suggested that lipoprotein transformations in man can occur through the S_{f20-10}^5 range by the progressive removal of triglyceride from the core of the large particles leading to smaller and smaller particles. Experimental evidence in support of the heparin catalysed transformations of the low density lipoproteins into lipoproteins of higher density has been provided by Shore and Shore (1962) and Lossow *et al* (1963). Shore and Shore (1962) concluded that the products of heparin induced lipolysis in human serum were low density lipoproteins with flotation values of between 11 and 20. The injection of cholesterol -4-^{14}C labelled rat chyle into heparin treated rats resulted in a high proportion of the label being found in the S_{f0-20} lipoproteins after 10 minutes incubation (Lossow *et al* 1963). It still remains to establish unequivocally whether or not the end products are identical with the plasma lipoproteins of the same flotation rates.

The results in this thesis show that as thoracic duct lymph chylomicrons from rabbits are acted upon by serum with clearing factor lipase *in vitro*, the overall turbidity of the solution decreases. However as can be seen in Table 17 there is an increase in optical density

of the VLDL and D>1.019 fraction at the same time as a decrease in the chylomicron fraction optical density. This suggests a transformation of the chylomicrons to more dense particles of smaller diameter.

Electron microscopy (Fig. 30) also suggests an increase in particles in the VLDL and D>1.019 fractions. It must be pointed out however that although comparable dilutions were made in each fraction it is difficult to be sure that the amount put on and blotted off each grid is the same. The appearance of L shaped particles was also noted in the VLDL and D>1.019 fractions of the clearing factor experiment. Whether these are artefactual or represent a large particle partially hydrolysed is unsure. The chylomicrons incubated with clearing factor serum appeared no longer spherical, were coalescing and surrounded in places by an electron dense film. These possibly represent chylomicrons undergoing hydrolysis.

The studies of the in vivo radioactively labelled moieties of thoracic duct lymph chylomicrons show that there is a decrease in both the fatty acid and cholesterol component of the chylomicrons when incubated with clearing factor in vitro (Tables 18 and 19). Most of the transferred radioactive fatty acid ends up in the D>1.019 fraction, presumably as a free fatty acid-albumin

complex; whereas much of the transferred ^{14}C-cholesterol is in the VLDL fraction, possibly representing smaller lipoproteins than the original chylomicrons due to hydrolysis of their triglyceride core. There is also some radioactivity from cholesterol in the D>1.019 fraction which may represent still smaller and more dense particles originating from the chylomicron fraction.

The chemical composition of the chylomicron fraction after incubation with normal serum and clearing factor serum is shown in Table 20. As would have been expected from the radioactive experiments there was a decrease in the triglyceride and cholesterol content of the chylomicrons when incubated with clearing factor. This decrease was more marked for the triglyceride component leading to a lower $\frac{TG}{C}$ ratio. In the chylomicrons after incubation with normal serum however there was also a slightly lower $\frac{TG}{C}$ ratio, more marked in those incubated for 24 hr at 37^{o}C. The decrease in triglyceride concentration is hard to explain, unless either some lipolysis is occuring in the control serum, or the ultracentrifugation of the serum-chylomicron mixture is inefficient. The increase in the cholesterol content might be due to a transfer of free cholesterol from the serum lipoproteins to the lymph chylomicrons

(Fredrickson et al, 1958, Goodman, 1962: Minari and Zilversmit, 1963; Zilversmit 1968a). Further studies of this problem would involve the measurement of free fatty acids and free and esterified cholesterol in both the chylomicron and subnatant fractions. The experiments with clearing factor lipase in this thesis do indicate that its action on thoracic duct lymph chylomicrons is to produce smaller particles, relatively more rich in cholesterol. The results therefore agree with the suggestion of French et al (1955) that the lipoproteins of rabbit serum may represent a failure in the disposal of cholesterol containing residue of the thoracic duct lymph chylomicrons.

The same possibility is suggested by the experiment in which labelled cholesterol was recovered from the subnatant fraction (VLDL and D>1.019) of rabbit serum after the intra-venous injection of labelled thoracic duct lymph chylomicrons. The results of this experiment resembled the in vivo experiments of Lossow et al (1963) in rats, except no heparin had been given to the rabbit.

2. The rate of disappearance of exogenous lipid from the blood stream

As mentioned in the Introduction the exchanges between the individual lipid components of lipoproteins

are complex so the study of the removal from the serum of exogenous lipid derived from the chyle is difficult. Although from the study of chyle by the electron microscope it is tempting to regard the chylomicrons and lipoproteins as morphological entities, it is apparent that once they enter the blood stream their individual components may follow different metabolic pathways. For example the half life of cholesterol in the blood stream of man is up to 60 days (Lewis and Myant, 1967), whereas the half life of low density lipoprotein is only 2-35 days (Walton *et al*, 1965; Fredrickson *et al*, 1967; Walton 1967).

As well as individual lipid components following different metabolic pathways, another difficulty is that they can leave and re-enter the circulation by various routes. For example, cholesterol may be excreted in the bile and be re-absorbed from the gut lumen; or a more rapid cycle may occur when the triglyceride component of chylomicrons is hydrolysed by enzymes such as clearing factor lipase to result in circulating lipoproteins rich in cholesterol (see previous discussion).

The removal of chylomicrons from the circulation *in toto* probably accounts for the rapid early phase of clearance from the blood stream (French and Morris, 1957,

1958). Few lipoproteins as large as chylomicrons escape *in toto* from the blood stream into peripheral lymph (Courtice and Morris, 1955; Morris and Courtice, 1955; Courtice and Garlick, 1962), but it is probable that they are trapped in organs such as the liver where their composite lipids then undergo differential metabolism (Borgström and Jordan, 1959; Dole and Hamlin, 1962; Belfrage, 1968; Jeanrenaud, 1968). Electron microscopic studies have supported this concept (Ashworth *et al*, 1960; Jones *et al*, 1966). However the triglyceride component of chylomicrons can still be removed from the blood stream in hepatectamised animals. Bragdon and Gordon, (1958) and Nestel *et al* (1962b) from studies in the removal and redistribution of labelled triglyceride in chylomicrons in dogs suggested that many tissues besides liver can directly remove the triglyceride of chylomicrons from the blood stream. Simpson-Morgan (1968) with an isolated rat heart-lung preparation found that this preparation could directly metabolize chylomicron triglyceride. He pointed out, however, that only in the liver and heart-lung preparation had direct proof of chylomicron triglyceride utilization been obtained, and that chylomicron triglyceride found in other organs (French and Morris, 1958) might be retransported

in the form of free fatty acid or other lipoproteins from the primary sites of removal.

The study of the removal of the triglyceride moiety of chylomicrons has shown that its circulating half life is short, in the order of minutes (Havel and Fredrickson, 1956; French and Morris, 1957, 1958; McCandless and Zilversmit, 1958; Morris, 1958; Nestel *et al*, 1962c; Morris and Simpson-Morgan, 1963, Simpson-Morgan, 1968).

The phospholipid component of chylomicrons is removed from the blood stream at rates probably different from triglyceride. The literature however is rather confusing since Havel and Fredrickson (1956) found the triglyceride and phospholipid components were removed at the same rate in dogs while McCandless and Zilversmit (1958) found phospholipid was removed more slowly than triglyceride.

Unlike the triglyceride of chylomicrons which is metabolised or stored in many tissues, the cholesterol of chylomicrons is probably removed from the blood stream by the reticulo-endothelial system, especially the liver (Friedman and Byers, 1954; French and Morris, 1958; Dole and Hamlin, 1962; Nestel *et al*, 1963; Fredrickson *et al*, 1967; Jeanrenaud, 1968). Not only

is the liver the major site of removal of exogenous cholesterol but it is also the major organ of excretion by way of the bile and bile acids (Bloch et al, 1943; Fredrickson et al, 1967; Dietschy, 1968). The half life of cholesterol in man has been found to be 52-62 days (Lewis and Myant, 1967) whereas exogenous triglyceride in man has been found to have a half life of only a few minutes (Nestel et al, 1962a; Hallberg, 1965). Nestel et al, (1963) found that cholesterol of thoracic duct lymph chylomicrons of dogs was removed more slowly than the triglyceride component in most, but not all, of the dogs studied. Quarfordt and Goodman (1966a and b) noted the cholesterol component of thoracic duct lymph chylomicrons was removed by the liver from the blood stream of rats considerably faster after the injection of large chylomicrons than after the injection of small chylomicrons. The subsequent pathway of metabolism of cholesterol, however, was the same from both large and small chylomicrons (Quarfordt and Goodman, 1967).

The results in this thesis suggest that after the intravenous injection of whole chyle labelled in vivo with either ^{14}C-cholesterol or ^{3}H-palmitic acid, the triglyceride leaves the blood stream of rabbits more

quickly than does the cholesterol (Tables 21 and 22, and Fig. 31). The disappearance of cholesterol from the blood stream of rabbits fed ^{14}C-cholesterol with diets of cholesterol-plain food or cholesterol-30 per cent oil, is also slow since at the end of 3 weeks much radioactivity still remained in the serum (Fig. 32).

There is some evidence that the size of chylomicrons might affect their rate of removal from the blood stream. French and Morris (1957) noted that the rate of removal of triglyceride of thoracic duct lymph chylomicrons from rat serum was faster than the removal of the triglyceride from the subnatant, which would be contained in smaller particles. The results of McCandless and Zilversmit (1958) however did not confirm this as little difference was found in the rate of the triglyceride or phospholipid components from the chylomicrons or subnatant fraction of thoracic duct lymph of dogs.

The removal of the cholesterol content of lipoproteins by the liver and reticulo-endothelial system may also depend to some extent on the size of the particle containing it in view of the findings of Quarfordt and Goodman (1966a and b, 1967). This difference in size was suggested as a possible reason for the hypercholesterolaemia of rabbits as compared with other

species fed cholesterol by Zilversmit et al (1967), in view of the finding that much cholesterol entered the blood stream in comparatively small VLDL particles of thoracic duct lymph in the rabbit. Fredrickson et al (1967) have also mentioned the importance of the size of particulate lipid in its removal from the blood stream as a possible explanation for the slower turnover of glycerides in pre-β lipoproteins as compared to the larger chylomicrons.

The findings in this thesis (Tables 21 and 22 and Fig. 31) show that both the triglyceride and cholesterol components of lipoproteins of the chylomicron fraction are removed from the blood stream more quickly than from the smaller lipoproteins of the VLDL fraction. The same recipient rabbit in some cases was used for both chylomicron and VLDL fractions, so animal difference in these cases was excluded. Another possible variable, the difference of lipid mass injected (French and Morris, 1957), was also excluded since the chylomicron fraction contained the most lipid, yet was still removed faster than the VLDL fraction. Another possibility that VLDL might enter the extra-cellular tissue fluid pool through the lymphatics more rapidly than chylomicrons (Courtice and Garlick, 1962) would tend to make the VLDL disappear

from the circulation more quickly than chylomicrons. The reverse however was found and although only a few animals were studied the evidence was that the larger chylomicrons were removed from the circulation more quickly than the smaller thoracic duct VLDL in the rabbit.

One anomaly seen in these experiments is that although for the whole lymph the triglyceride fraction leaves the blood stream faster than the cholesterol fraction, this difference is not so apparent when the chylomicron and VLDL fractions are compared. One possible explanation of this is that after ultracentrifugal separation, the lipoproteins are no longer treated by the rabbit as physiological but as foreign bodies. This possibility might explain the differences in the findings of Havel and Fredrickson (1956) and McCandless and Zilversmit (1958) since the lipoproteins separated by the former workers possibly were treated as foreign bodies but those of the latter workers, because of some difference in separation technique, still behaved as 'physiological' particles.

The literature, although rather conflicting, and the results of this thesis indicate that chylomicrons are removed from the blood stream faster than the smaller lipoproteins of the VLDL fraction of chyle. Further

experiments to clarify this proposition however seem indicated. For example, the labelling of different moieties of chylomicrons at the same time with, for example, ^{3}H-triglyceride ^{14}C-cholesterol ^{32}P-phospholipid and ^{131}I-protein might be possible, and different techniques of ultracentrifugation might prevent lipoproteins from becoming 'foreign'. The presence or absence of bromide ions in ultracentrifugation media, for instance, might be a factor in altering the chylomicrons. Finally the preparation of chylomicrons of different diameter by the feeding of different triglyceride loads might be a method of preparing physiological particles of different sizes without the need of ultracentrifugation.

3. <u>The effect of dietary fat load on serum cholesterol levels in rabbits</u>

The results shown in Table 15 suggest that serum cholesterol levels in rabbits ingesting cholesterol and plain food are higher than in rabbits eating the same quantity of cholesterol, but with high loads of dietary triglyceride. This phenomenon was again looked at and, as can be seen from Table 24 and Fig. 33, the resultant hypercholesterolaemia is markedly reduced in those groups of rabbits eating 30 per cent triglyceride concurrently with the cholesterol, whether the

triglyceride be corn oil, butter, Frymasta or soya bean oil. The serum levels of those rabbits fed 15 per cent corn oil or butter were intermediate between the low and high fat diets.

As has been seen in the Methods there is from 3-5 per cent triglyceride present in the plain rabbit food; so it was also decided to examine serum cholesterol levels of rabbits fed the same quantity of cholesterol in ether-extracted (lipid free) food, plain food and food to which an extra 5 per cent corn oil or butter had been added. Table 24 shows that the serum cholesterol levels of rabbits fed ether-extracted food did rise, but not as markedly as when plain food was fed. Similarly the serum cholesterol levels of rabbits fed 5 per cent added butter or corn oil was slightly higher than when plain food was added. In an analysis of variance on these results, however, Dr Simpson-Morgan (1968, personal communication) found the difference was not statistically significant between the cholesterol-plain food groups and cholesterol-5 per cent added triglyceride groups. (Fig. 33).

Thus it can be seen that when rabbits are fed the same amount of cholesterol daily, with an equal caloric intake but with varying triglyceride loads, if no

triglyceride is fed the serum cholesterol levels do not rise as high as when small amounts of triglyceride are ingested. However when the proportion of triglyceride is increased to 15 or 30 per cent the subsequent serum cholesterol levels are diminished.

The explanation of these results is not known but there are a number of possibilities. Firstly, the high loads of triglyceride might prevent or decrease the absorption of cholesterol; secondly, the presence of other sterols such as phytosterols might interfere with the absorption of cholesterol; thirdly, as has been seen earlier, the size of the lipoprotein-containing cholesterol in thoracic duct lymph before entry into the blood stream might influence its rate of removal from the blood stream; fourthly the previous proposition or the increased triglyceride load might cause an increased excretion of cholesterol by the liver in the form of bile and bile acids; and finally, the triglyceride load might influence the synthesis of cholesterol in the rabbit intestine or liver. These explanations make the findings difficult to explain and point the way to further experiments, but a discussion of these possibilities will be attempted.

The feeding of cholesterol to rabbits has long been used to produce hypercholesterolaemia (Pribram, 1906) and the deposition of lipid in the arterial wall (Anitschkow, 1933). Most experiments have included the feeding of triglyceride at the same time but Popják (1946) showed that cholesterol fed as a fine aqueous emulsion without added triglyceride was absorbed by the rabbit. This might be due to the presence of endogenous fatty acids from the breakdown of cellulose within the gut lumen or from the phospholipids of bile (Shrivastava et al, 1967). It is recognised that triglyceride and its breakdown products associated with micelles of bile acids are necessary for the absorption of cholesterol in mammals, but that cholesterol favours the emulsion phase rather than the micellar phase and so is not absorbed as readily as triglyceride (Borgström, 1960; Hofmann and Borgström, 1962; Simmonds et al, 1967; Borgström, 1968; Sylvén and Borgström, 1968).

Borgström (1968) in the rat showed that when increasing amounts of cholesterol were fed in a constant amount of triolein the percentage of cholesterol absorbed decreased only gradually while the total amount absorbed increased. At the lowest dose fed only 50 per cent of the dietary cholesterol was absorbed even though the amount fed led

to a 10 to 15 fold increase in total absorption. Borgström suggested that the rapid and complete absorption of triglyceride and subsequent transit of the intestinal content to the large intestine were probably important factors in determining the extent of absorption of nonglyceride fat. Sylvén and Borgström (1968), again in the rat with constant triglyceride ingestion, fed varying amounts of cholesterol. They found an almost constant fraction of dietary cholesterol (0.4) was recovered in 24 hr irrespective of the dose fed from a trace up to 100 μmoles in 800 μmoles triolein. They suggested that cholesterol absorption had the characteristics of a passive diffusion process. A possible explanation of the decreased serum cholesterol levels found in rabbits eating high triglyceride diets might be small intestinal hurry, (Bennett and Simmonds, 1962; Bennett, 1964), causing cholesterol to reach the large bowel before absorption.

The second explanation of lower serum cholesterol levels in rabbits on high triglyceride diets might be the presence of phytosterols in the diet leading to reduced absorption. This mechanism was suggested by Peterson *et al* (1953) in chickens, and Beveridge *et al*

(1958) and Malmros (1958) in man, as one explanation of the hypocholesterolaemic effect of vegetable oils.

However, Bloomfield (1964) with rats fed 0.64 per cent cholesterol with 20 per cent butter or safflower oil noted that those fed safflower oil had an increased cholesterol absorption of 30-40 per cent compared with butter fed rats. Borgström (1968) in the rat found that β-sitosterol was absorbed in amounts only about one tenth of the corresponding dose of cholesterol and that the feeding of sitosterol with cholesterol seemed to have the same effect on cholesterol absorption as feeding the same additional dose of cholesterol. In view of the subsequent findings of Sylvén and Borgström (1968) it seems that large doses of phytosterol would be necessary to decrease cholesterol absorption in the rat. Aramaki et al (1967), in studies on the effect of cholestane -3 , 5 , 6 -triol in reducing serum cholesterol levels in rabbits, chickens and rats, found that 0.5 per cent. β-sitosterol would only slightly decrease the serum cholesterol level rise in rabbits fed 0.5 per cent cholesterol.

The decrease in rise of serum cholesterol in rabbits fed triglyceride in this thesis (Fig. 33 and Table 24) is unlikely to be due to phytosterol since,

as can be seen in the Methods, the amount of phytosterol fed varied greatly from one fat diet to another, yet all triglycerides tested kept the serum cholesterol levels low. For example, the phytosterol intake of rabbits fed 400 mg cholesterol daily varied from about 35 mg to 165 mg of phytosterol daily depending on whether the rabbits were fed 30 per cent butter or 30 per cent corn oil; and was about 70 mg phytosterol per day in those rabbits eating cholesterol and plain food.

Thirdly the more rapid removal of exogenous cholesterol from the blood stream when rabbits are fed cholesterol with a high triglyceride load is a possible explanation. As already seen (Fig. 25, 27, 28) cholesterol enters the blood stream from thoracic duct lymph in larger particles in rabbits fed cholesterol and 30 per cent triglyceride than when fed cholesterol and plain food. Friedman and Byers (1954) suggested that the most likely explanation of the hypercholesterolaemia of rabbits was the poor removal of exogenous cholesterol from the circulation by the reticulo-endothelial system and the liver as compared with other animals. It is possible in view of the findings in this thesis that the rabbit's liver might be better able to remove

cholesterol from larger lipid particles than from smaller particles (Fig. 31).

It should be pointed out that the feeding experiments described in this thesis only extended over a few weeks since it was found that the livers of rabbits fed cholesterol, especially when fed cholesterol and high fat diet, were grossly pathological at the end of 12 weeks. Since, as discussed earlier, the liver plays a dominant role in removal and excretion of cholesterol it was felt that only rabbits early in the experiment would give physiological results. An example of the histology of livers from two typical rabbits is shown in Fig. 38. The livers of cholesterol + low fat fed rabbits exhibited cholesterol crystals (bi-refrigent to polarised light and with a positive Schultz stain) around the centre and mid zone of the liver lobules, but with most liver cells still normal. The livers of those animals fed cholesterol and 30 per cent triglyceride (butter or corn oil) exhibited marked centri-lobular necrosis, large lipid laden cells towards the periphery and fibrosis around the bile ducts. Few bi-refrigent crystals were present in these livers but cholesterol staining (Schultz) was apparent around the bile ducts.

Liver pathology, and hence the inability to remove exogenous cholesterol from the serum, might explain the apparent difference in the results shown in this thesis and those found by Van Handel and Zilversmit (1959), who noted that after five months the serum cholesterol levels of rabbits fed cholesterol and 20 per cent cotton seed oil were higher than of those fed cholesterol with low fat diet. It can be seen in their experiments, however, that earlier the serum cholesterol levels of the fat fed rabbits were lower than in the animals on the low fat diet, and the extent of atherosclerosis observed after five months was also slightly less in the animals on the high fat diet.

The excretion of cholesterol might also be a factor in the difference noted between the groups of rabbits studied in this thesis. Fig. 34 shows the original serum levels of rabbits made hypercholesterolaemic by a three week diet of 400 mg cholesterol in plain food and the subsequent changes in serum cholesterol when various triglycerides were added to the diet. It can be seen that in the rabbits in which cholesterol feeding was suspended there was a progressive decrease in serum cholesterol levels. This decrease, however, was more rapid when 30 per cent corn oil was added to the diet.

Bailey and Butler (1967) noted that semi-starvation slowed the decrease in serum cholesterol levels after cholesterol feeding had ceased in hypercholesterolaemic rabbits. One possible explanation of the above findings is that triglycerides stimulate bile flow and so cholesterol excretion by the liver, either in the form of cholesterol or bile acids (Klevay and Hegsted, 1968). Another possibility is that large chylomicrons entering the blood stream from the thoracic duct might take up cholesterol from the smaller serum lipoproteins, either as an exchange phenomenon (Zilversmit, 1968) or by adsorption of small lipoproteins onto chylomicrons (Courtice and Garlick, 1962; Furman et al, 1962) so enabling the liver to remove the resulting larger cholesterol containing particles from the circulation.

Fig. 34 also shows that in hypercholesterolaemic rabbits, if the same amount of cholesterol is still fed, but if 30 per cent triglyceride is added to the diet, the hypercholesterolaemia lessens in the rabbit on the high fat diet. It will be seen that 37 per cent butter was added in this case since preliminary observations showed that 30 per cent butter and cholesterol did not drop the serum cholesterol as quickly as 30 per cent corn oil. Since butter is not 100 per cent butter fat

(see Methods) it was decided to feed a higher quantity of butter. Even at this level, however, the butter did not cause as marked a decrease in serum cholesterol level as did corn oil. The explanation of this is unknown unless the extra cholesterol from butter (about 40 mg per day) might cause the observed difference, or that bile secretion varied as to the type of fat fed (Klevay and Hegsted, 1968).

The same dose of ^{14}C-cholesterol was fed to two rabbits on a cholesterol-plain food diet and two rabbits on a cholesterol-30 per cent corn oil diet. As can be seen in Fig. 32 the serum radioactivity in the rabbits eating the plain food reached higher levels and remained higher than in those on the high triglyceride diet. The cause of this also might be decreased absorption, increased excretion or altered distribution of cholesterol within the rabbits' body.

As regards the final possibility that triglyceride in the diet slows cholesterol synthesis, little can be said. Gould and Taylor (1950) have shown that cholesterol synthesis in the liver of rabbits, as in dogs, is diminished when cholesterol is fed and Popják and Beeckmans (1950) have shown that the intestine of the rabbit synthesises cholesterol. Dietschy and Wilson (1968)

have shown that bile salts alter the rate of intestinal cholesterol synthesis. If cholesterol synthesised in the intestine is carried in the VLDL fraction of thoracic duct lymph, as Wilson and Reinke (1968) have suggested in the rat, then perhaps the decreased VLDL cholesterol seen in the thoracic duct lymph of rabbits fed cholesterol-high fat diet (Fig. 27 and 28) might represent decreased cholesterol synthesis.

In summary it can be seen that cholesterol fed with high triglyceride loads to rabbits results in decreased serum cholesterol levels when compared with rabbits fed cholesterol and low triglyceride loads. The explanation is not known but one difference observed was the size of the cholesterol carrying lipoprotein entering the blood stream from thoracic duct lymph (Fraser and Courtice, 1968). It will be necessary in future experiments to standardize not only the amount of cholesterol fed but also the triglyceride intake and, owing to subsequent liver damage, results should be obtained in the first few weeks of the experiment. At present Dr West in this department is setting up gas-chromatographic methods which will be used in cholesterol balance studies to try to determine the role

of absorption and excretion of cholesterol and its breakdown products in rabbits fed cholesterol with and without concurrent triglyceride.

E. ATHEROSCLEROSIS

1. General discussion

Atherosclerosis has been a problem for medical research workers for many years with as yet few answers about its aetiology, prevention or cure.

The study of the role of serum lipids in the cause of atherosclerosis followed Aschoff's findings that atheromatous plaques contained cholesterol and the observations of Anitschkow on "experimental atherosclerosis" in rabbits (cf. Gould, 1951; Blumenthal, 1967). Stimulus to the study of the lipid component of serum came from Gofman and his associates (1950) who noted that cholesterol containing lipoproteins of specific physical chatacteristics were associated with human coronary heart disease. So widespread became the interest in the constituents of blood that Professor Duff was moved to write that one might get the impression that the disease process was so independent of the substrate of the vessel wall that it might occur in the absence of the blood vessels themselves (cf. Whereat, 1959).

One of the theories of the origin and development of atherosclerosis in man postulates that the earliest change is a splitting or reduplication of the intimal elastic lamina, beginning in early life and progressing to a well-developed musculo-elastic subendothelial layer by early adulthood (Wilens, 1951; Movat et al, 1958, Moon and Rinehart, 1952; Schornagel, 1956; Stehbens, 1960; Buck, 1963; Osborn, 1963). The lack of correlation between the presence of lipid in these early changes supports the view that deposition of lipid is not the initiating factor. Although the presence of lipid is usually a striking feature of the advanced atherosclerotic lesion, the precise role that the plasma lipids play in this disease is still far from clear. The intimal fatty streak, appearing in the first decade of life as an intracellular accumulation of lipid in the smooth muscle cells of the subendothelial layer, is thought by some workers to progress to form atherosclerotic plaques when the lipid becomes extracellular (Holman et al, 1958; Geer et al, 1961; McGill et al, 1963).

It is fairly generally agreed that the ultimate severity of the lesion depends, in part at least, upon the level of cholesterol in the plasma (Gofman, 1959;

Gofman and Young, 1963; Dawber et al, 1962); this suggests that lipid deposition may occur by a process of filtration of the plasma lipoproteins (Page, 1954).

The early degenerative lesions that occur in the elastic tissue of arteries in man have also been shown to occur in a large variety of animals and to lead to areas of intimal thickening - for example, in primates (Mann et al, 1953; Lindsay and Chaikoff, 1957; Cox et al, 1958; McGill et al, 1960), dog (Lindsay et al, 1952), cat (Lindsay and Chaikoff, 1955), pig (Gottilieb and Lalich, 1954; French et al, 1963), rabbit (Stehbens, 1963) and birds (Lindsay et al, 1955; Clarkson et al, 1959). Although lipid is sometimes seen in these lesions, it is not usually a predominant feature, unless the plasma cholesterol level is increased by dietary or other means (Gould, 1951; Roberts and Strauss, 1965).

Morphologically similar focal lesions may be induced experimentally in animals by various types of arterial injury (Ramsey et al, 1936; Waters, 1953; Taylor, 1955; Prior and Hartman, 1956; Williams, 1961; Courtice and Schmidt-Diedrichs, 1962, 1963; Lindsay, Entenman et al, 1962; Lindsay, Kohn et al, 1962). The primary lesion in these circumstances seems also to be a fragmentation or splitting of the internal elastic lamina with subsequent

fibrocellular thickening of the intima. With the animal on a normal diet, little or no lipid is found in the lesion. However, when cholesterol is added to the diet to produce hypercholesterolaemia, lipid is rapidly deposited in the thickened intima. On the other hand, when prolonged hypertriglyceridaemia is produced by repeated administration of the surface-active agent, Triton WR 1339, lipid is not deposited (Courtice and Schmidt-Diedrichs, 1962, 1963). These experiments suggest that certain physical or chemical characteristics of plasma lipoproteins may be important in the process of lipid deposition.

Another experimental lesion, the primary lipid-type of atherosclerosis, referred to in this thesis as "experimental atherosclerosis", has for many years been shown to develop in animals by an initial deposit of foam cells beneath the endothelium without preliminary intimal thickening, when the cholesterol level in the plasma is increased by one of various methods (Leary, 1941; Duff and McMillan, 1951). The origin of the foam cell is uncertain: some workers maintain it reaches the vessel from the macrophages of the blood (Leary, 1941; Duff et al, 1957; Sparagen et al, 1962; Gonzales, 1963; Gonzales and Furman, 1965). Others feel it is derived

from the endothelium (Osborn, 1963; Pollak, 1965), while still others think it is a transition from smooth muscle cells or other cells of the media (Constantinides, 1965; Imai et al, 1966; Parker and Oland, 1966; Charman and Lipsky, 1967; Knieriem et al, 1967; Wissler, 1967). More than one source of foam cell has also been suggested (Koide et al, 1963; Day, 1967). Changes in the elastic tissue with the formation of a fibrocellular subendothelial layer seem, in these conditions, to be secondary to the primary lipid deposit.

Until the natural history of human atherosclerosis is fully studied, which pathological change comes first will remain uncertain (Osborn, 1963; Constantinides, 1965). Certainly the end product of intermittent cholesterol feeding in the rabbit as produced by Constantinides (1965) is very similar in appearance to human atherosclerosis in that it contains much fibrosis and extracellular cholesterol.

The experimental lesion in animals does not develop, however, when an experimental hyperlipaemia is produced by Triton, cortisone or alloxan. Furthermore, when any one of these agents is administered to cholesterol-fed rabbits, the development of this primary lipid-type atherosclerosis is considerably inhibited (Duff and

McMillan, 1949; Kellner et al, 1951; Payne and Duff, 1951; Billiau et al, 1963; Wang et al, 1955; Butkis et al, 1968). The repeated injection of heparin in the cholesterol fed rabbit prevents the subsequent turbidity of the serum, diminishes the extent of atherosclerosis, but does not alter greatly the serum cholesterol levels (Constantinides et al, 1953).

It seems probable, therefore, with regard to arterial lipid deposition, either secondary to naturally-occurring or experimentally-induced intimal thickening, or primary as in the primary lipid-type lesion, that different types of hyperlipaemia in animals have different effects. These observations led Professor Courtice and his team in this department to study the properties of plasma lipoproteins in experimentally-induced states (Garlick and Courtice, 1962; Courtice and Munóz-Marcus, 1964), and of the mechanisms concerned in the transference of the lipoproteins across the vascular endothelium (Courtice and Garlick, 1962; Courtice et al, 1964). The results of their experiments suggest that both the size and composition of the plasma lipoproteins are important factors in their transfer across the endothelial lining and their deposition in the intima. They also showed that the broad spectrum of lipoproteins

in plasma of hypercholesterolaemic rabbits closely resembled the patterns of the lipoproteins in man (Garlick et al, 1965).

Although lipids are also known to be synthesised within the arterial wall (Chernick et al, 1949; Siperstein et al, 1951; Zilversmit et al, 1954; Whereat, 1967; Day, 1967), or to result from the breakdown of red blood cells or platelets from small haemorrhages from the vasa vasorum (Osborn, 1963; Paterson, 1967), or mural thrombi (Rokitanski, 1852; Duguid, 1955; Mitchell and Schwartz, 1965; Benko and Laki, 1968), it is unlikely that these sources of lipid account for more than a fraction of the cholesterol found in atheromatous plaques (Friedman and Byers, 1964; Newman and Zilversmit, 1964; Day, 1967; Adams, 1967). It has been suggested, however, that the synthesis of lipid within the artery wall is a protective mechanism in that fatty acids and phospholipid under the influence of enzymes convert the deposited free cholesterol to cholesterol esters, which are less sclerotic and aid in its subsequent removal (Adams et al, 1963; Lofland et al, 1965; Day, 1967; Adams et al, 1967; Adams, 1967).

It is generally believed that most of the cholesterol in atheromatous lesions is derived from the circulating cholesterol of plasma lipoproteins and furthermore some

workers suggest that the level of serum cholesterol derived from exogenous rather than endogenous sources is correlated with the production of experimental atherosclerosis in the rabbit (Duff and McMillan, 1949; Duff and Payne, 1950; Biggs and Kritchevsky, 1951; Byers and Friedman, 1954), in the dog (Gonzalez et al, 1959), and in the rat (Dayton et al, 1961). It thus seemed pertinent to examine lipoproteins of the thoracic duct before entry into the blood stream and the development of experimental atherosclerosis in the cholesterol-fed rabbit.

2. Specific discussion

As already seen, the cholesterol of chyle is contained in larger lipoproteins from rabbits fed a high diet of fat with cholesterol than when fed cholesterol and a low fat diet. It has also been shown that the extent of hypercholesterolaemia is less in rabbits ingesting the high fat diet, although a causal relationship between this and lipoprotein size has not been proved. Fig. 35 and Table 25 show that not only is hypercholesterolaemia reduced in cholesterol-fed rabbits on a high fat diet but also the incidence of experimental atherosclerosis is decreased. Whether the reduction of

experimental atherosclerosis is related to the size of the cholesterol containing lipoproteins entering the circulation, and/or to the low serum cholesterol levels, is uncertain (Fraser and Courtice, 1968).

That the dietary vehicle in which cholesterol is fed to rabbits influences the extent of experimental atherosclerosis has been the subject of many studies. Most workers since Anitschkow used some form of triglyceride, usually a vegetable oil, in combination with cholesterol. However, as pointed out by Popják (1946), ingested fat is not essential for cholesterol absorption in the rabbit. Widely varying levels of serum cholesterol and extent of experimental atherosclerosis have been reported in the literature, and recently some attention has been focussed on the food with which cholesterol is fed. Kritchevsky and his group have been actively interested in this problem and have compared various diets and oils for their atherogenic effects (Kritchevsky et al, 1954, 1956, 1962; Kritchevsky, 1961; Kritchevsky and Tepper, 1967, 1968). They found that the feeding of 6-9 per cent oil with cholesterol increased the serum cholesterol levels and atherogenicity, especially if the oil had previously been heated. Although slight differences of serum

cholesterol levels were noted between saturated and unsaturated oils by the former workers, Kobernick et al (1964) concluded that saturated fats in the diet were not a major factor in the pathogenesis of experimental atherosclerosis in the rabbit.

Most workers, however, did not study the effects of triglyceride in as high proportions as used in this thesis. Van Handel and Zilversmit (1959), on the other hand, studied rabbits fed cholesterol with and without 20 per cent cottonseed oil. As stated earlier, although the animals on the high fat diet at the end of five months had higher serum cholesterol levels, early in the experiment the level was lower. The extent of atherosclerosis was slightly lower in the high fat group at the end of five months feeding despite the terminal higher serum cholesterol levels.

On examining the literature it seems that cholesterol when fed with a low fat diet has a high atherogenic effect. It is recognised, however, that other variables such as diet, age, weight, breed and exercise of rabbits make these comparisons uncertain (Pollak, 1965; Kobernick and Hashimoto, 1963). Pollak (1945) fed cholesterol powder in capsules to rabbits eating normal food and produced comparatively high

levels of serum cholesterol. Altschul (1946), by feeding rabbits cholesterol made of baked cakes from egg yolk, and keeping the dietary fat to a minimum, produced atherosclerosis in only 19 days. Lipid deposition occurred also in the choroid plexus, when previously it had been found that cerebral vessels were not affected in the cholesterol-fed rabbit. Firstbrook (1950) noted that semi-starvation increased the atherogenicity of cholesterol. Yuschenko (1959) described greatly accelerated production of atherosclerosis from feeding cholesterol to rabbits with grated carrots rather than with vegetable oils.

A correlation between serum cholesterol levels and atherosclerosis has been shown to exist in the results of this thesis. The serum cholesterol levels of rabbits fed 30 per cent fat plus cholesterol were lower than those fed cholesterol and plain food (Tables 15 and 24, Fig. 33) and the extent of experimental atherosclerosis was also less in the cholesterol-high fat group of rabbits (Table 25, Fig. 35). A correlation between serum cholesterol levels and experimental atherosclerosis had previously been noted (Anitschkow, 1933; Pollak, 1945; Duff and McMillan, 1951; Kobernick et al, 1964), but, as stated by Newman and Zilversmit (1964), the

duration of hypercholesterolaemia is also important. However, as discussed earlier, physical or chemical changes in the lipoproteins carrying the serum cholesterol may alter this relationship. It has been shown that surface active agents, although not lowering the serum cholesterol, prevent the development of experimental atherosclerosis (Kellner et al, 1951; Billiau et al, 1962, in the rabbit; and Butkis et al, 1968, in the dog). It is interesting to note that Garlick et al (1965) suggested that the size of lipoproteins resulting from Triton WR 1339 injections in the rabbit were larger than the usual lipoproteins of hypercholesterolaemic rabbit serum.

The actual mode of entry of serum cholesterol into the arterial wall is not known, although an exchange of the various lipid and protein components of lipoproteins of the serum has been demonstrated (National Heart Foundation of Australia, 1967; Adams, 1967; Hollander, 1967; Smith et al, 1967; Wissler, 1967). One suggestion is that the complete lipoprotein passes through the endothelial barrier, and is known as the filtration theory.

The filtration theory has some support from the findings of Courtice and Morris (1955) and Courtice and

Garlick (1962), who have shown that lipoproteins can pass through the endothelium of peripheral blood vessels into lymph. There is a greater escape of the smaller lipoproteins from the circulation. The gradient of labelled albumin and lipids within arteries has also been examined and lends some support to this theory (Buck, 1955; Duncan et al, 1959; Duncan and Buck, 1960, 1961). Although not proven, the filtration theory has much to recommend it in that it gives a rationale to the atherogenicity of certain subfractions of lipoproteins (Gofman et al, 1950) and could possibly explain the findings in this thesis. However absolute evidence of the passage of the complete lipoprotein across the endothelium has yet to be found. The further study of this problem will probably involve labelling of the various moieties of the lipoprotein, electron microscopy and biochemical and metabolic studies.

One objection to the filtration hypothesis, however, is that although it is suggested that the smallest diameter lipoproteins filter most easily into the vessel wall, cholesterol in the clear subnatant fraction, representing probably endogenous cholesterol, does not produce experimental atherosclerosis in the rabbit. For example, Bragdon (1951), in transfusion transfer of

experimental atherosclerosis in the rabbit, found that only the light scattering lipoprotein of the serum were atherogenic, while the clear subnatant containing small lipoproteins with cholesterol did not produce lipid deposits in the artery. This same point has been suggested by Byers and Freedman (1954) in explaining that only exogenous cholesterol in the serum is atherogenic.

3. A hypothesis

A tentative explanation of the findings of this thesis or modification of the filtration theory might depend on four concepts.

Firstly, the lipoproteins entering the circulation for the first time from the thoracic duct differ not only chemically from the lipoproteins found in the serum of the hypercholesterolaemic rabbit (Table 16), but are also the potentially atherogenic particles, unlike the "burnt out" secondary serum particles.

Secondly, the fate of the cholesterol of the "primary" particles depends on their size. The cholesterol of the larger particles is removed from the blood stream, possibly by the reticulo-endothelial system or liver, to be stored or excreted. The smaller particles are dealt with differently, either to become "burnt out" secondary

particles by hydrolysis of their triglyceride, or to a lesser extent incorporated into arterial walls. This second point suggests that in the rabbit "experimental atherosclerosis" and hypercholesterolaemia are both the result of small "primary" cholesterol containing lipoproteins reaching the blood stream; and not that "experimental atherosclerosis" is the result of hypercholesterolaemia per se.

Thirdly, from a flow dynamics or rheological viewpoint, a low speed region exists close to the walls of arteries in which "primary" particles could temporarily reside. The diameter of the primary particle has importance in that, if large, the more the particle would project into the more energetic central region. The larger particles would thus tend to be swept along with the stream, whereas the small particles would have a better chance of remaining for some time in contact with the vessel wall. The thickness of the low speed region will vary at bends and branches of arteries, becoming thicker where streamlines are wide apart, as for example distal to a branch, so favouring the longer residence of "primary" particles.

Fourthly, the arterial wall contains enzymes, including lipases and phospholipases, which can act on

primary lipoproteins temporarily in contact with the endothelium, causing hydrolysis of the triglyceride component, so leaving a still smaller cholesterol-rich particle which if not swept away becomes incorporated within the vessel wall.

Evidence in favour of this hypothesis is that it has been shown in this thesis that cholesterol containing lipoproteins from the thoracic duct are larger when rabbits are fed triglyceride in high doses, and that the development of experimental atherosclerosis is reduced.

That lipid deposition in the artery wall is probably related to blood flow and streamlining is well known. It can be seen from Fig. 36 that lipid deposition occurs below and lateral to intercostal branches in the aorta, and later above these vessels deposition does not take place. This theory has been used previously to explain atherogenesis in terms of mural thrombi or vessel damage (Murphy et al, 1962; Fox and Hugh, 1966; Mitchell and Schwartz, 1965; Rodbard, 1959; Texon, 1960). It might equally be used to suggest a contact of lipoproteins with the artery wall in areas of a thick boundary layer.

The arterial wall is known to contain lipases and phospholipases (Gresham and Howard, 1962; Leites, 1965;

Patelski et al, 1967, 1968) which might act on the lipoproteins in contact with the endothelium. Schoefl and French (1968) with electron microscopy have demonstrated chylomicrons adhering to capillary walls in the lactating mammary gland and suggested that these were being acted on by lipase.

The hypercholesterolaemic plasma containing cholesterol-rich particles, mainly of VLDL size, would not be acted on by this mechanism since they probably represent "burnt out" particles already dealt with by the liver or other organs, and are not affected by clearing factor lipase (French et al, 1955, and personal observations). Duff et al, 1957, observed early lipid deposits just deep to the endothelium of the aorta in rabbits fed 1 per cent cholesterol only 4-16 hr before autopsy, and consequently with normal serum cholesterol levels.

4. The relationship of atherosclerosis in the rabbit to that in man

Whether the study of "experimental atherosclerosis" in the rabbit has any bearing on the human form of the disease is unknown. As pointed out by Duff and McMillan (1951), great caution should be exercised in transposing interpretations derived from studies of experimental

cholesterol atherosclerosis into terms supposedly applicable to the human disease. The rabbit is a popular experimental model since it rapidly develops "experimental atherosclerosis" and its use will no doubt continue. Many schools, however, are changing to other animals more closely resembling man in eating habits or appearance.

Pollak (1958) in an argument against the use of the rabbit as an experimental model pointed out that cholesterol is not a normal part of the rabbit's diet (although rabbits occasionally eat eggs), large amounts of cholesterol are fed to the experimental animals, the level of hypercholesterolaemia is far higher than is seen in man, and the lesions involve mainly the ascending and thoracic aorta. The lesions are xanthomatous and resemble the fatty streak lesion seen in humans rather than true atherosclerosis.

However in 1965 Pollak was impressed with a new concept of "episodic polygenic atherogenesis". This followed work by Constantinides (1965) who produced lesions in the rabbit artery closely resembling human lesions. These included fibrosis and necrosis brought about by long term episodic cholesterol feeding. Pollak also pointed out that even small amounts of dietary

cholesterol, or only 5 mg/Kg of intravenous colloidal cholesterol, could bring about arterial lesions. The huge doses of oral cholesterol and acute florid foam cell lesions produced by most workers in rabbits are probably a result of impatience.

Osborn (1963), from the study of extensive autopsy material, attempted to map out the natural history of coronary atherosclerosis in man. This also appears to follow an "episodic polygenic" course. However, as Robert Koch pointed out to his students, "Meine Herren, vergessen Sie nie, dass die Mäuse keine Menschen sind".

SUMMARY

This thesis mainly dealt with the transport of ingested lipid in thoracic duct lymph, its subsequent transport and removal from the blood stream and its association with experimental atherosclerosis in the cholesterol-fed rabbit. A comparison between the size and composition of chylomicrons and lipoproteins was undertaken since both their physical and chemical properties probably have importance in their metabolism.

1. Rabbits were the main experimental animals studied because of their susceptibility to experimental atherosclerosis. The thoracic duct lymph from rats was also compared with that from rabbits.

2. Various triglycerides including corn oil and butter were fed to animals in differing concentrations. In some cases cholesterol, with and without added triglycerides, was also fed and the sterol content of the diets estimated to determine the concentration of phytosterols.

3. The collection of biological samples included the development of a relatively simple technique of thoracic duct cannulation in the rabbit.

4. The lipoproteins of serum and thoracic duct lymph were fractionated in the preparative ultracentrifuge

into very low density lipoproteins (VLDL) of $S_{f12-400}$, chylomicrons of $S_{f>400}$, and lipoproteins of density greater than 1.019 g/ml (D>1.019) which included low density lipoproteins of S_{f0-12} and high density lipoproteins.

5. An electron microscopic method for the measurement of the diameter distribution, surface area and volume of a random sample of lipoproteins was designed. This method was found to be suitable only for those chylomicrons and lipoproteins containing unsaturated lipid since osmium was used for fixation. A freeze-etching method for the electron microscopic preparation of chylomicrons from butter-fed animals was attempted and preliminary electron micrographs shown.

6. As well as the size of the chylomicrons and lipoproteins, their lipid composition was determined by chemical analysis.

7. It was found that the chylomicrons in thoracic duct lymph of corn oil and cholesterol fed rabbits were mostly larger than 720Å in diameter but rarely larger than 5,000Å, the VLDL ranged from 360-840Å, while the D>1.019 lipoproteins were smaller, the maximum diameter being about 600Å with many lipoproteins of about 120Å in diameter. Although some overlap in particle size

between the fractions was observed the division between the smallest chylomicrons and largest VLDL approximately coincided with the theoretical size based on physical data from ultracentrifugation.

8. The size of chylomicrons in thoracic duct lymph was found to increase on high triglyceride dietary loads. The size increase was determined by electron microscopy in corn oil fed animals. In rabbits on the low fat diet the mean diameter of chylomicrons was 960Å with half the triglyceride in particles of diameter greater than 1400Å; on the high fat diet the mean diameter was 1435Å with half the triglyceride in particles of diameter greater than 2700Å. In the rat the mean chylomicron diameter was 1094Å in the early phase of fat absorption with half the triglyceride in particles of diameter greater than 1320Å. These figures increased to 2105Å and 3600Å respectively at the peak of fat absorption and subsequently decreased to 1004Å and 1200Å toward the end of fat absorption. No marked difference in the size of thoracic duct lymph VLDL from rabbits fed varying corn oil diets was noted. The mean diameter of VLDL was 524Å from the rabbits fed the low fat diet and 504Å from the rabbits fed the high fat diet.

Indirect evidence from the TG/PL ratios of the chylomicron fraction of chyle from rabbits fed 5 and

30 per cent butter suggested that these likewise increased in size on the high fat diet. Electron microscopic confirmation of this has yet to be obtained.

9. The comparison of the surface area/volume ratio with the triclyceride/phospholipid ratio of chylomicrons of various sizes and VLDL from the thoracic duct lymph of animals fed varying triglyceride loads was compatible with the hypothesis that triclyceride makes up the bulk of the core or volume of the particles, and phospholipid is evenly spread in a thin layer on the surface. Each phospholipid molecule was calculated to occupy an average area of about 65Å^2, suggesting that the phospholipid was distributed as a monomolecular layer.

10. The diameter distribution of lipid particles in various artificial fat emulsions was compared with chylomicrons. It was found that if manufactured at a slightly alkaline pH, and with a similar TG/PL ratio to chylomicrons, the diameter distribution of particles was comparable with chylomicrons. The sterol content of emulsions was also examined and it was suggested that phytosterols found to be present might explain some of their toxic reactions.

11. It was found in cholesterol-fed rabbits that relatively more cholesterol in the thoracic duct lymph

was carried in larger lipid particles when a cholesterol-high fat diet was fed than after a cholesterol-low fat diet. The mean diameter of chylomicrons from rabbits fed 0.8 per cent cholesterol - 30 per cent corn oil was 1476Å, while that from rabbits fed 0.8 per cent cholesterol in plain food was 1065Å. The mean diameters of the VLDL were 493 and 499Å respectively.

Not only were chylomicrons larger in the cholesterol-high fat diet group of rabbits but cholesterol in the thoracic duct lymph was also distributed to a greater extent in this fraction. In the cholesterol-low fat diet group, on the other hand, relatively more cholesterol was present in the smaller VLDL.

12. In some experiments chylomicrons and lipoproteins were labelled <u>in vivo</u> by feeding rabbits ^{14}C-cholesterol or ^{3}H-palmitic acid and harvesting their thoracic duct lymph.

13. The comparison of cholesterol estimated chemically, and the distribution of radioactivity, following the ingestion of ^{14}C-cholesterol in the three fractions of thoracic duct lymph, suggested that the lipoproteins of D>1.019 were to a large extent derived by filtration from the serum, that some of the cholesterol of VLDL

was derived from sources other than ingested cholesterol, and that cholesterol of chylomicrons was derived in the main from the intestine.

14. The lipoproteins of serum from cholesterol-fed rabbits were examined. Unlike thoracic duct lymph, little lipid was present in the chylomicron fraction of serum. In hypercholesterolaemic rabbits the VLDL fraction contained the greatest amount of cholesterol. The serum of hypercholesterolaemic rabbits was observed to be turbid and, unlike lymph, the turbidity was not due to chylomicrons but to VLDL. It was found that the VLDL of rabbit serum were larger in diameter than of chyle, with diameters ranging up to 1440$\mathring{A}$.

While triglyceride was found to be the major lipid component of chylomicrons and VLDL from thoracic duct lymph, it was found that cholesterol was the major component of these fractions in serum.

15. Post heparin serum containing clearing factor lipase was found to alter chylomicrons from thoracic duct lymph <u>in vitro</u> by hydrolysing triglyceride, so resulting in smaller particles. This was studied by following the distribution of ^{3}H-palmitic acid and ^{14}C-cholesterol labelled chylomicrons after incubation with clearing factor lipase. Most of the ^{14}C-label

was redistributed to the VLDL fraction, while the ^{3}H-label entered the D>1.019 fraction, presumably bound to serum albumin as free fatty acid. Optical density and electron microscopic studies supported this view. The action of clearing factor lipase was suggested as one possible cause for the observed difference between lipoproteins of thoracic duct lymph and those present in serum.

16. Cholesterol was shown to leave the blood stream of the rabbit more slowly than triglyceride after the injection of radioactively labelled whole chyle. Both cholesterol and triglyceride were shown to leave the circulation more quickly following the injection of chylomicrons than after the injection of the smaller VLDL. It was suggested, however, that the fractionated lipoproteins might be treated as 'foreign' rather than physiological particles. Further experiments were suggested to check this finding.

17. Higher serum cholesterol levels were obtained in rabbits fed cholesterol and low fat diet than when fed the same amount of cholesterol daily but with high triglyceride loads. The rabbits were all fed 400 mg. of cholesterol daily. The triglycerides were either corn oil, soya bean oil, butter or 'Frymasta' which

were added in concentrations of up to 30 per cent. The rabbits on the cholesterol-low fat diet at the end of 3 weeks developed mean serum cholesterol levels in excess of 1000 mg per 100 ml whereas in those on cholesterol-high fat diets the serum cholesterol levels reached only 138-203 mg per 100 ml.

Serum cholesterol levels of rabbits previously made hypercholesterolaemic were shown to reduce more quickly if the animals were fed diets rich in triglyceride. The level of serum cholesterol could also be lowered even if cholesterol continued to be fed, but fed concurrently with 30 per cent corn oil or butter. Corn oil appeared to have a greater effect in reducing serum cholesterol levels than a similar high dose of butter.

Possible explanations of these findings were discussed and included suggestions that cholesterol was not absorbed so readily when fed with large doses of triglyceride, that cholesterol was excreted at a higher rate when triglyceride was fed and finally the size of the cholesterol containing lipoprotein entering the blood stream from the thoracic duct lymph might alter its subsequent fate and metabolism. It was thought unlikely that ingested phytosterols were responsible for the observed differences in these experiments.

18. The aortae and coronary arteries of rabbits fed cholesterol with and without triglyceride were examined for lipid deposition. It was found that rabbits fed cholesterol and 30 per cent corn oil or butter did not develop atherosclerosis to the same extent as those rabbits fed cholesterol and plain food. Possible explanations of this finding were discussed including a hypothesis linking rheology, the size of cholesterol containing lipoproteins entering the circulation from thoracic duct lymph, and the observed distribution of lipid deposition in the aorta.

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